STUDIES ON A DISINTEGRIN AND METALLOPROTEINASE WITH THROMBOSPONDIN MOTIFS-1, -4 AND -5 AND THE REGULATION OF THEIR GENE EXPRESSION IN MACROPHAGES

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A thesis presented for the degree of Doctor of Philosophy

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Abstract

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are a family of proteins that are closely related to the matrix metalloproteinases (MMPs). It has been suggested that the proteins have a critical role in the breakdown of articular cartilage during osteoarthritis (OA). More recently it has been suggested that their actions could potentially regulate atherosclerotic plaque stability.

Atherosclerosis is a chronic, inflammatory disorder characterised by lipid and cholesterol accumulation and the development of fibrotic plaques within the walls of large and medium arteries. The stability of the plaques is very important because clinical symptoms are only presented after rupture of the unstable plaques, leading to thrombosis and ischemia.

During the current study, immunohistochemical analysis confirmed that ADAMTS-1, -4 and -5 were being expressed within human carotid atherosclerotic lesions; macrophages were identified as major contributors to their expressions. Following on from this THP-1 macrophages were stimulated with transforming growth factor-β (TGF-β), interferon-γ (IFN-γ), TNF-like protein 1A (TL1A), interleukin (IL)-17A and IL-33. The regulation of ADAMTS-1, -4 and -5 expressions were analysed using quantitative polymerase chain reactions (QPCR) and western blots. It was shown that TGF-β increased the expressions of ADAMTS-1 and -5 and decreased the expression of ADAMTS-4. IL-33 decreased the expressions of ADAMTS-1, -4 and -5 and IFN-γ also decreased the expression of ADAMTS-1. TL1A and IL-17A stimulation of macrophages had no regulatory actions over ADAMTS-1, -4 or -5 expressions.

Looking at evidence from previous studies, TL1A and IL-17A were identified as agents that could potentially act in synergy to amplify pro-inflammatory cytokine responses. To investigate this further, THP-1 macrophages were stimulated with TL1A and IL-17A, TL1A and IFN-γ and also IL-17A combined with IFN-γ. TL1A and IL-17A were shown to act in synergy to increase the expressions of ADAMTS-1, -4 and -5 in macrophages.

The regulation of ADAMTS-1, -4 and -5 expressions in macrophages by IL-33 was studied further. The mechanism of signal transduction was studied using RNA interference (RNAi) targeting extracellular signal-regulated kinases (ERK)-1, ERK-2, p38, c-Jun N-terminal kinases (JNK)-1/2, c-Jun, phosphoinositide 3-kinase (PI3K)-γ, PI3K-δ, p50, p65 and Janus kinase (JAK)-1/2. It was determined that the attenuation of ADAMTS-1, -4 and -5 expressions occurred through transcriptional regulation that was dependent on the ST2 receptor. ERK-1, ERK-2, JNK-1/2, c-Jun, PI3K-γ and PI3K-δ were also involved in the signal transduction of the response.

The cellular roles of ADAMTS activity within atherosclerotic disease progression remain poorly understood. During the current study adenoviral vectors were created that delivered shRNA-targeting ADAMTS-1, -4 and -5. The adenoviral vectors were utilised in studies designed to investigate the roles of ADAMTS-1, -4 and -5 during macrophage migration and foam cell formation. The studies showed that knockdown of ADAMTS-1, -4 and -5 had no effect on macrophage migration or foam cell formation.

More research is required into the cellular roles that ADAMTS proteases play during atherosclerotic disease progression. The field of research is now growing and could potentially provide some exciting opportunities for novel therapeutics of the future.

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Publications

1. MCLAREN, J.E., MICHAEL, D.R., **ASHLIN, T.G**. and RAMJI, D.P., 2011. Cytokines, macrophage lipid metabolism and foam cells: Implications for cardiovascular disease therapy. *Progress in Lipid Research*, 50(4), pp. 331-347.
2. MCLAREN, J.E., MICHAEL, D.R., SALTER, R.C., **ASHLIN, T.G**., CALDER, C.J., MILLER, A.M., LIEW, F.Y. and RAMJI, D.P., 2010. IL-33 reduces macrophage foam cell formation. *Journal of Immunology*, 185(2), pp. 1222-1229.
3. MICHAEL, D.R., **ASHLIN, T.G**., BUCKLEY, M.L. and RAMJI, D.P., 2012. Liver X receptors, atherosclerosis and inflammation. *Current Atherosclerosis Reports*, 14(3), pp. 284-293.
4. MICHAEL, D.R., **ASHLIN, T.G**., BUCKLEY, M.L. and RAMJI, D.P., 2012. Macrophages, lipid metabolism and gene expression in atherogenesis: A therapeutic target of the future? *Clinical Lipidology*, 7(1), pp. 37-48.
5. SALTER, R.C., ARNAOUTAKIS, K., MICHAEL, D.R., SINGH, N.N., **ASHLIN, T.G**., BUCKLEY, M.L., KWAN, A.P.L. and RAMJI, D.P., 2011. The expression of a disintegrin and metalloproteinase with thrombospondin motifs 4 in human macrophages is inhibited by the anti-atherogenic cytokine transforming growth factor-β and requires Smads, p38 mitogen-activated protein kinase and c-Jun. *International Journal of Biochemistry and Cell Biology*, 43(5), pp. 805-811.
6. SALTER, R.C., **ASHLIN, T.G**., KWAN, A.P.L. and RAMJI, D.P., 2010. ADAMTS proteases: Key roles in atherosclerosis? *Journal of Molecular Medicine*, 88(12), pp. 1203-1211.

Abbreviations

Abbreviation	Full Term
ABC	ATP binding cassette transporter
ACAT	Acetyl-Coenzyme A acetyltransferase 1
AcLDL	Acetylated LDL
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADRP	Adipose differentiation related protein
Adz	Adenovirus with zero cloning steps
Akt	Protein Kinase B
AP	Alkaline phosphatase conjugate
AP-2	Activator protein-2
ATF2	Activating transcription factor 2
apoE	Apolipoprotein E
BAC	Bacterial artificial chromosome
BLAST	Basic Local Alignment Search Tool
BM	Basement membrane
CD68	Cluster of differentiation 68
Cdc42	Cell division control protein 42
ChIP	Chromatin immunoprecipitation
CINC	Chemokine-cytokine induced neutrophil chemoattractant
COMP	Cartilage oligomeric matrix protein
CPT-1	Carnitine palmitoyltransferase I
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCL	Chemokine ligand
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DR3	Death receptor 3
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Elk-1	E twenty-six (ETS)-like transcription factor 1
ERK	Extracellular signal-regulated kinases
ET-1	Endothelin
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor

FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HB-EGF	Heparin-binding epidermal growth factor
HDL	High density lipoprotein
HI-FCS	Heat inactivated FCS
HMDM	Human monocyte derived macrophage
ICAM-1	Intercellular adhesion molecule-1
IDL	Intermediate density lipoprotein
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor
IL	Interleukin
IMT	Intima-media thickness
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
LDL	Low density lipoprotein
LTBP	Latent TGF-β binding protein
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
MIF-1	Macrophage migration inhibitory factor
MIP-1	Macrophage inflammatory protein-1
MMLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
NADPH	Nicotinamide adenonine dinucleotide phosphate
NCEH	Neutral cholesterol ester hydrolase
NF-1	Neurofibromatosis type I
NFκB	Nuclear factor κ-light chain enhancer of activated B-cells
NF-AT	Nuclear factor of activated T-cells
NO	Nitric oxide
NPC-1	Niemann-pick disease, type C1
OA	Osteoarthritis
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDK1	Pyruvate dehydrogenase lipoamide kinase isozyme 1
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PPAR	Peroxisome proliferator activated receptor
QPCR	Quantitative PCR
RA	Rheumatoid arthritis
RCT	Reverse cholesterol transport
RIP	Receptor interacting protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
Runx	Runt-related transcription factor
RXR	Retinoid X receptor
SARA	Smad anchor for receptor activation
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
Sp1	Specificity protein 1
SR-A	Scavenger receptor-A
STAT	Signal transducer and activator of transcription
TBE	Tris/borate/EDTA
TGF-β	Transforming growth factor-β
T_h	T-helper
TIMP	Tissue inhibitor of metalloproteinases
TL1A	TNF-like protein 1A
TNF-α	Tumour necrosis factor-α
TNFR	TNF receptor
TRADD	TNF receptor type 1-associated death domain protein
TRAF	TNF receptor-associated factor
TRITC	Rhodamine
TWEAK	TNF-like weak inducer of apoptosis
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
WHO	World Health Organisation

Chapter 1

Introduction – The role of ADAMTS-1, -4 and -5 during atherosclerosis

1.1 – Atherosclerosis Pathology

1.1.1 – Background

Statistics from the World Health Organisation (WHO) indicate that more people die annually from cardiovascular disease (CVD) than any other cause. In 2004, an estimated 17.1 million people died from CVD, representing nearly 29% of all global mortalities. Of these, an estimated 7.2 million were due to coronary heart disease and 5.7 million were due to stroke. The WHO has predicted that by 2030 almost 23.6 million people will die from CVD-related illness worldwide (McLaren et al. 2011). In 2009 alone, it was estimated that CVD cost the US economy $475.3 billion (Roger et al. 2012). The major clinical complications that are associated with atherosclerosis are heart attack, stroke and gangrene. The prevalence of mortality is only predicted to rise due to developing countries acquiring westernised lifestyles and increasing worldwide levels of obesity and diabetes. These statistics and predictions highlight the importance of future atherosclerosis research in the hope of developing new therapeutic advances.

Atherosclerosis is considered to be a progressive disease that is characterised by lipid accumulation and inflammation within the walls of the large and medium arteries (McLaren et al. 2011). During the progression of the disease lesions develop in the vascular wall that are termed atherosclerotic plaques. The formation of the plaques are considered to be linked with chronic inflammation, and changes to the physiological functions of the various cell types within the vascular wall (Lusis 2000). The different stages of atherosclerosis are outlined and briefly explained in Figure 1.1.

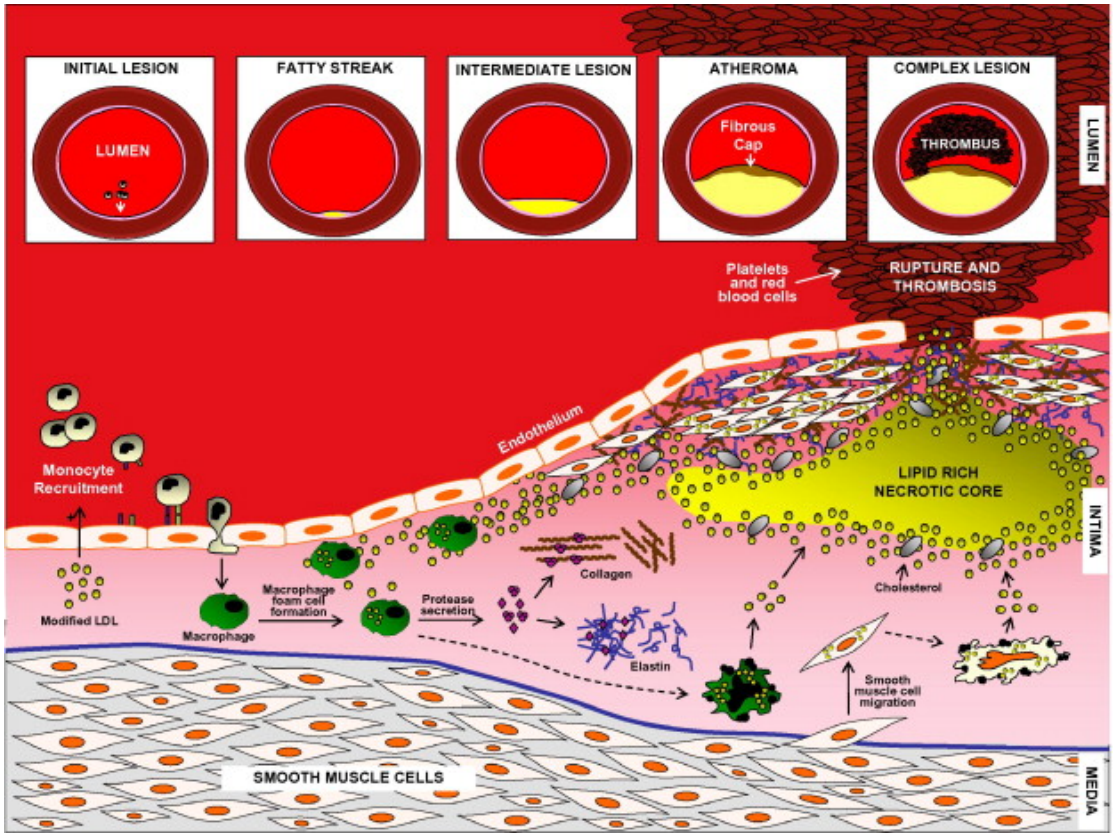


Figure 1.1: **Overview of atherosclerosis progression (McLaren et al. 2011)**

Fatty streak formation is initiated by recruitment and infiltration of surrounding blood monocytes, which subsequently differentiate into macrophages. Macrophages take up modified low density lipoprotein (LDL) from the plaque surroundings to produce lipid laden foam cells. Further progression is observed when vascular smooth muscle cells (VSMC) migrate and proliferate to form a fibrous cap. As the plaque progresses extracellular matrix (ECM) remodeling via protease expression and apoptosis/necrosis of foam cells modulate the stability of the mature plaque. Further escalation of atherosclerosis centres on increasing inflammation and increasing vulnerability of the plaque to rupture.

1.1.2 - Risk Factors

Atherosclerosis has a very complex aetiology; there are many genetic and environmental risk factors that interact to contribute to disease progression (Table 1.1). The large numbers of risk factors outline the increasing importance for clinicians to be able to predict patient specific cardiovascular endpoints using biomarkers. Previous tests aiming

to do this looked at the intima-media thickness (IMT) and found a correlation between IMT and the occurrence of fatal cardiovascular events (Simon et al. 2002). The protocol for measuring the IMT was complicated and erroneous, so new biomarkers of atherosclerosis were sought. Physicians have now turned to measuring levels of serum biomarkers such as C-reactive protein (CRP) (Elgharib et al. 2003). During atherosclerosis the levels of CRP are elevated around 1000-fold (Corrado et al. 2010). Despite the success of CRP as an atherosclerotic marker predictions on the stability of the plaque cannot be achieved (McLaren et al. 2011). A search for novel biomarkers identified MMPs as a potential marker for plaques that are vulnerable to rupture (Newby 2006). Serum levels of pregnancy-associated plasma protein-A, a zinc binding MMP, have been shown to increase when plaques become vulnerable to rupture (Kajinami and Kawai 2011; McLaren et al. 2011). More recently a study has found another serum biomarker of premature cardiovascular events, Wnt-1. Lower levels of Wnt-1 were observed in patients that had suffered myocardial infarction (Goliasch et al. 2012). The availability of these biomarkers for atherosclerosis could lead to patient specific treatment programmes to try and slow and even reverse the progression of atherosclerosis.

Table 1.1: Risk factors associated with atherosclerosis (Lusis 2000).

Type	Risk Factor
Genetic	Elevated LDL/very low density lipoprotein (VLDL) Reduced levels of high density lipoprotein (HDL) Elevated blood pressure Elevated levels of homocysteine Family history Type I diabetes and obesity Elevated levels of haemostatic factors Depression Gender (male) Systemic Inflammation Metabolic syndrome
Environmental	High fat diet Smoking Low anti-oxidant levels Lack of exercise Infectious agents Type II diabetes

Both genetic predisposition and environmental factors have been shown to increase the risk of atherosclerosis. Genetic predisposition can commonly be linked to a patient's family history of cardiovascular events. Environmental factors are largely linked to patient lifestyle.

1.1.3 - Lipids in atherosclerosis

As mentioned in the previous section (1.1.2), elevated levels of LDL and VLDL in the bloodstream have been identified as major risk factors for atherosclerosis. The importance of aberrant lipid metabolism within the initiation and progression of atherosclerosis makes it important to understand how lipids are metabolised under normal physiological conditions (Figure 1.2). Cholesterol is a sterol that is essential for membrane fluidity; most of the cholesterol in the body is formed by *de novo* synthesis

but can also be consumed in the diet (Ikonen 2006). Cholesterol can either be transported to the peripheral tissues or the liver, or can accumulate in intracellular stores (Ikonen 2006). The pathway that is important for cholesterol excretion is reverse cholesterol transport (RCT) (van der Velde 2010). RCT functions by utilising a HDL-dependent mechanism to help remove un-wanted cholesterol from the peripheral tissues by transporting it to the liver for excretion (van der Velde 2010). Cholesterol is transported in the blood stream within water-soluble lipoprotein particles. These lipoprotein particles are characterised by their density and the various types perform different functions. The different types of lipoprotein are presented in Table 1.2 where their roles in cholesterol trafficking are also described. An overview of cholesterol metabolism is presented in Figure 1.2.

Table 1.2: Lipoprotein particles and their role in cholesterol transport (McLaren et al. 2011)

Lipoprotein Particle	Role in Lipid Metabolism
Chylomicron	Carry absorbed triacylglycerol and cholesterol from the intestine, to the liver, adipose tissue and skeletal muscle.
VLDL	Carry triacylglycerols synthesised in the liver to the peripheral tissues.
Intermediate Density Lipoprotein (IDL)	Intermediate between VLDL and LDL; level in blood is very low.
LDL	Functions to carry cholesterol from the liver to peripheral tissues.
HDL	Reverse cholesterol transport; functions to carry cholesterol from peripheral tissues back to the liver.

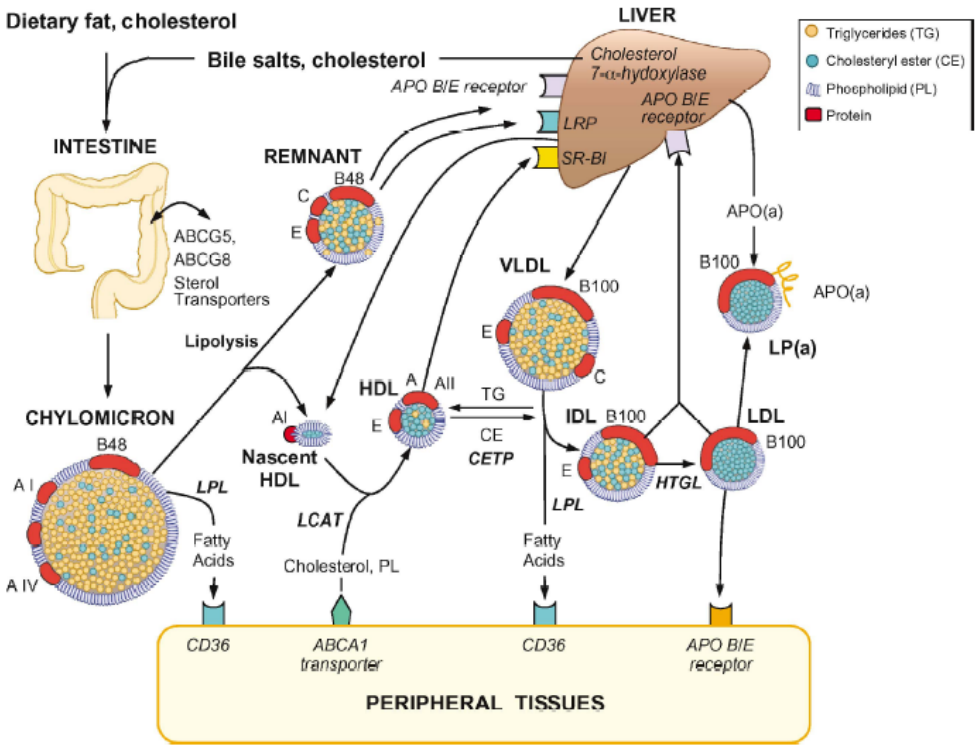


Figure 1.2: **Lipoprotein metabolism and transport (Lusis et al. 2004)**

The pathway of absorption from the intestine is shown, with chylomicrons transporting the triacylglycerol and cholesterol to the liver and the peripheral tissues after absorption from the diet. Also LDL is shown to carry cholesterol from the liver to peripheral tissues, and HDL is shown to carry out reverse transport back to the liver. HDL= High Density lipoprotein. LDL = Low Density Lipoprotein. IDL = Intermediate Density Lipoprotein. VLDL = Very Low Density Lipoprotein.

1.1.4 - Monocyte recruitment, macrophages and fatty streak formation

An outline of fatty streak formation is presented in Figure 1.3, followed by a series of detailed descriptions below.

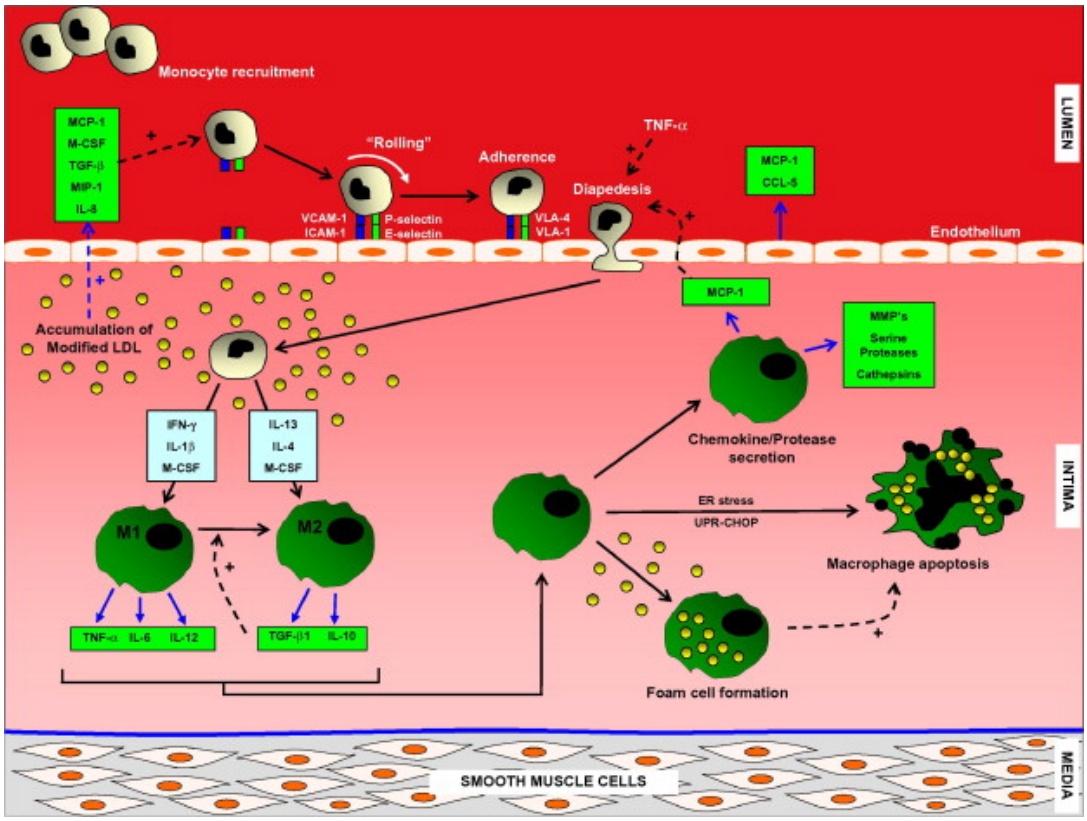


Figure 1.3: Plaque initiation and fatty streak formation (McLaren et al. 2011)

Firstly, the endothelial cells become activated and express chemoattractant molecules for monocytes. The monocytes then interact with the molecules such as the selectins, VCAM-1 and ICAM-1, causing monocyte adherence. The monocytes then migrate into the subendothelial space. Here they are able to differentiate into macrophages with distinct subsets M1 and M2, and begin to express markers of macrophage differentiation. The macrophages undergo foam cell formation and produce chemokines and proteases leading to increased plaque progression and inflammation.

1.1.4.1 – Plaque Initiation

One of the early signs of atherosclerosis is the appearance of fatty streak lesions within the intima of medium and large arteries (Lusis 2000). The fatty streak is not a clinically

significant lesion as it cannot directly cause acute events such as heart attack or stroke, although the lesion can progress into a mature plaque (Halvorsen et al. 2008). According to the modified 'response to injury' hypothesis of atherosclerosis, endothelial damage, stress or activation leads to adherence, followed by migration of monocytes into the sub-endothelial matrix (Bobryshev 2006; Ross 1993). Activation of the endothelial cells (ECs) is the main initiating factor of atherosclerosis, with accumulation of LDL in the sub-endothelial matrix being the major cause. The prevalence of activation is further aggravated by other risk factors such as high blood pressure and smoking (Lusis 2000). Once activated, the ECs express various chemoattractant molecules including moncyte chemoattractant protein-1 (MCP-1) that attract monocytes in the blood stream to the injury site, this process leads to the formation of fatty streaks in these locations (McLaren et al. 2011). Monocytes adhere to selectin molecules on the surface of the endothelial cells. P- and E-selectin molecules are expressed on the luminal surface of the activated endothelium and they interact with L-selectin molecules on the surfaces of monocytes (Bobryshev 2006). The interaction between the selectins cause monocytes to roll across the endothelial surface, which enables molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) to initiate binding with integrins (Bobryshev 2006). Integrin binding causes a higher level of adhesion to the endothelium so that the migration through the endothelial layer to the intima can now begin. Diapedesis is aided by the expression of chemokines by endothelial cells such as MCP-1, macrophage colony stimulating factor (M-CSF), macrophage inflammatory protein-1 (MIP-1), tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (Bobryshev 2006; Lusis 2000).

1.1.4.2 – Monocyte – macrophage differentiation

Once the monocytes have passed through the endothelial layer by diapedesis and are present in the intima of the blood vessels, differentiation can take place (Lusis 2000). Depending on the local inflammatory environment the monocytes can either differentiate into macrophages or dendritic cells (Bobryshev 2006). The main regulator of the differentiation process is M-CSF. Many genes associated with macrophage differentiation begin to be expressed such as apolipoprotein-E (apoE), scavenger receptors and cluster of differentiation 68 (CD68) (McLaren et al. 2011).

1.1.4.3 – Macrophage heterogeneity

As mentioned in the previous section, the local environmental concentrations of inflammatory mediators can dictate the direction of cell lineages (section 1.1.4.2). The local concentration of certain cytokines and growth factors can also control heterogeneity amongst the macrophage cell population within the plaque (Gordon and Martinez 2010; Shimada 2009). The majority of macrophages present within the plaque accumulate modified LDL to become lipid laden foam cells (section 1.1.4.4), however some do not. This suggests there could be different roles for different subsets of plaque residing macrophages (Wilson 2010). Different classifications of macrophage subsets have been presented: monocyte to macrophage differentiation could result in the pro-inflammatory, classically activated (M1) macrophages, or the anti-inflammatory, alternatively activated (M2) macrophages (Martinez et al. 2009). Both M1 and M2 macrophages have been shown to reside in the atherosclerotic plaque and an imbalance of the M1:M2 ratio could cause increased plaque progression (Hirata et al. 2011; Khallou-Laschet et al. 2010; McLaren et al. 2011). Cytokines play a major role in polarising the macrophage population, T helper (T_h)1 cytokines such as IFN-γ promote M1 macrophages and T_h2 cytokines such as IL-4 promote M2 macrophages (Martinez 2011). The macrophages display plasticity between the subsets and factors such as cytokine stimulation and activation of peroxisome proliferator activated receptor (PPAR)-γ can cause a phenotypic switch (Chawla 2010; Martinez 2011). This plasticity was further outlined when additional subsets of macrophage where identified called M4, Mox and Mhem (Boyle et al. 2012; Gleissner et al. 2010). The exact roles of the macrophage subsets during atherosclerosis are very poorly understood and the role of T_h1 and T_h2 responses during polarisation of the macrophage population could prove critical in the design of future cytokine therapies aimed to slow or reverse the progression of atherosclerosis (McLaren et al. 2011).

1.1.4.4 – Macrophage foam cell formation

During the development of an atherosclerotic lesion LDL begins to accumulate in the sub-endothelial matrix (Lusis 2000). Under normal conditions the cellular uptake of LDL is mediated by the LDL receptor (Soutar and Naoumova 2007). The expression of the LDL receptor is regulated via a negative feedback loop. If there is a high concentration of LDL

then the expression of the LDL receptor will be reduced, therefore protecting the cell from being over loaded with cholesterol (Soutar and Naoumova 2007). One particular characteristic of atherosclerosis is the formation of lipid laden macrophage foam cells within the lesion (Bobryshev 2006). One of the major contributors to foam cell formation is the uncontrolled macrophage uptake of modified-LDL particles from the subendothelial matrix via scavenger receptors which are not under negative feed-back regulation via intracellular cholesterol levels (Lusis 2000). Possible LDL modifications include acetylation and most predominately oxidation (Miller et al. 2010). Many mechanisms exist to oxidise LDL, including transition metals such as divalent cations, haem, as well as a number of different enzyme systems, such as lipoxygenases, myeloperoxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and nitric oxide (NO) synthases (Miller et al. 2010; Tsimikas and Miller 2011). The major proposed mechanism by which the macrophages take up the modified-LDL is scavenger receptor-mediated (McLaren et al. 2011; Moore and Freeman 2006). There are many scavenger receptors that can contribute to foam cell formation but the major two that have been extensively studied are scavenger receptor (SR)-A and CD36 (Bobryshev 2006). Extensive work using mouse models of atherosclerosis have shown that when scavenger receptor expression is reduced, on a whole the extent of atherosclerotic lesions were reduced (Lusis 2000). The deletion of SR-A or CD36 mainly resulted in a marked decrease in atherosclerotic lesion size and arterial lipid accumulation in hyperlipidemic mouse models (Bobryshev 2006). However, a study by Moore et al. (2005) showed that atherosclerosis was not reduced by knockout of SR-A or CD36, this result could suggest that there are also alternative LDL uptake mechanisms that could contribute to foam cell formation (Bobryshev 2006; Moore et al. 2005). Although native LDL appears to require modification to become a high-affinity ligand for scavenger receptors, enzymatic modifications by sphingomyelinase, phospholipase C, or secretory phospholipase-A2 can lead to increased retention of lipoproteins by matrix proteoglycans and internalisation by alternative uptake pathways (Moore and Freeman 2006). Furthermore, native LDL has been reported to be taken up into macrophages via macropinocytosis of extracellular fluid (Moore and Freeman 2006). The uptake of modified-LDL in the very early stages of atherosclerosis is considered to be a protective measure due to the removal of cytotoxic and inflammatory modified-LDL. As the disease progresses modified-LDL uptake is correlated with the

growth of the atherosclerotic lesion therefore acting in favour of pathology (Bobryshev 2006). Thoroughly understanding the mechanisms of how macrophages uptake cholesterol during atherosclerosis could be an important step in designing future therapeutic interventions.

Once the modified LDL has been taken up, it is transported within vesicles to the lysosomes and finally gets stored in the cytoplasm as cholesterol esters or triglyceride rich lipid droplets (Chinetti-Gbaguidi and Staels 2009). A high level of esterified cholesterol in the cytoplasm is a good indicator of a macrophage foam cell. A wide network of proteins tightly regulates the mechanism of cellular cholesterol storage. These include Niemann-Pick disease type C (NPC)-1, NPC-2, carnitine palmitoyltransferase (CPT)-1, adipose differentiation related protein (ADRP), acetyl-coenzyme A acetyltransferase (ACAT)-1 and neutral cholesterol ester hydrolase (NCEH) (Chinetti-Gbaguidi and Staels 2009; McLaren et al. 2011). NPC-1 and -2 control the trafficking of cholesterol from the lysosome ready for efflux, CPT-1 reduces cholesterol esterification and ADRP increases triglyceride storage (Chinetti-Gbaguidi and Staels 2009). The aberrant cellular storage and trafficking of cholesterol during atherosclerosis is very important because if cholesterol is not prepared for efflux in the normal physiological way, then foam cell formation will be enhanced, increasing the progression of atherosclerosis (McLaren et al. 2011).

Cholesterol efflux by RCT is a mechanism that clears cholesterol from cells for hepatic removal; however during atherosclerosis this machinery is dampened causing intracellular cholesterol deposition (McLaren et al. 2011). Members of the ABC transporter family such as, ATP-binding cassette transporter (ABC)A-1 and ABCG-1 are involved in directing cholesterol efflux from macrophages (Lusis 2000). The process of RCT requires the presence of lipid-free, cholesterol acceptors like apoE, the cholesterol is then transported back to the liver via HDL particles (Greenow et al. 2005). HDL particles are protective against atherosclerosis and act in two ways to inhibit the formation of early fatty streak lesions. Firstly, HDL has antioxidant properties that enable less oxidated-LDL to be present in the lesion, therefore slowing foam cell formation (Lusis 2000). The antioxidant properties arise from paraoxonase which is an enzyme carried on

HDL (Lusis 2000). Also, the HDL acceptor acts to remove excess cholesterol from peripheral tissues by transporting it away back to the liver via an ABCA-1 dependant mechanism (Figure 1.2). The important involvement of ABCA-1 in the regulation of cholesterol accumulation is clear in Tangier disease patients. The mutated form of ABCA-1 these patients possess is unable to help in the efflux of cholesterol leading to excessive macrophage cholesterol levels (McLaren et al. 2011; Oram 2000). The importance of cholesterol efflux in the progression of atherosclerosis is further demonstrated by the use of apoE$^{-/-}$ mice as a model for the disease (Imaizumi 2011; Nakashima et al. 1994; Pendse et al. 2009; Plump et al. 1992). These mice quickly develop atherosclerosis when fed on a high fat diet, underlining the importance of trying to reduce the accumulation of LDL within macrophages during atherosclerosis. Another common model system for atherosclerosis within mice is the LDL receptor null mice fed on a high fat diet; again this model further highlights the importance of elevated serum LDL concentrations in the development of atherosclerosis (Ishibashi et al. 1993; Ishibashi et al. 1994).

1.1.4.5 – Important roles of macrophages/foam cells in atherosclerosis

In the atherosclerotic plaque where macrophage egress is defective, the residing macrophages can partake in other significant roles in disease progression other than foam cell formation (McLaren et al. 2011). One important function they perform is to produce chemokines and cytokines that can orchestrate further monocyte infiltration and T-cell regulation, thereby amplifying the immune response (Charo and Taubman 2004). Macrophages are also able to secrete proteases that remodel the plaque ECM. This ECM remodelling can regulate various cellular actions, including VSMC migration, proliferation and apoptosis (Newby 2008). The many important roles that macrophages play within the plaque, especially orchestrating the immune response, have led to a vast amount of research into their regulation during the various stages of atherosclerosis.

1.1.5 - Smooth muscle cell migration and plaque progression

As the disease progresses, the fatty streak lesions usually begin to be covered by a fibrous cap, made up of VSMC and ECM components including elastin, collagen and versican (Halvorsen et al. 2008). The fibrous cap encloses a developing lipid-rich necrotic core, which usually contains debris from apoptotic and necrotic foam cells. Macrophage foam cells undergo apoptosis, particularly in advanced atherosclerotic lesions where free cholesterol accumulation in foam cells triggers apoptotic signals such as the endoplasmic reticulum stress pathway (McLaren et al. 2011; Tabas et al. 2009). During the pathology of atherosclerosis the efferocytosis of apoptotic macrophages is also reduced leading to an increased level of cellular debris within the necrotic core of the lesion (McLaren et al. 2011; Tabas 2010).

Migration and proliferation of VSMC are very important in the formation of the fibrous cap, and the process is highly regulated by various factors (further reviewed in section 1.1.6). However, the growth factors alone are not the only regulatory feature, macrophages also secrete proteases which remodel the ECM promoting fibrous cap formation (Newby and Zaltsman 1999). Studies have shown that an interaction between CD40 and its ligand is very important in the development of advanced lesions (Lusis 2000; Mach et al. 1997b; Schönbeck et al. 2000). CD40 is expressed on macrophages and T-cells and its purpose is to promote the expression of growth factors and cytokines that stimulate the migration and proliferation of VSMCs. The interaction between CD40 and CD40L also causes matrix degrading proteases to be released that remodel the ECM (Lusis 2000; Nagashima et al. 2004). The formation of the fibrous cap causes the complex lesion with a large necrotic core to become more stable. The stability of the plaque is maintained by having a balance between ECM synthesis and breakdown, if the balance is lost then the stability of the plaque is lowered (McLaren et al. 2011). A plaque in this chronic pro-inflammatory unstable condition is at high risk of rupture (Halvorsen et al. 2008). It has been of great research interest to elucidate the mechanisms by which a plaque becomes unstable during atherosclerosis. The prospect of plaque stabilising therapies could be a very important advance in trying to reduce the burden that atherosclerosis has on society.

1.1.6 - Plaque stability

1.1.6.1 – VSMC migration

During atherosclerosis different cell types, including ECs, platelets and macrophages produce growth factors and cytokines that regulate VSMC migration (Newby 2006). VSMCs usually reside in the medial layer of blood vessels, they provide strength and support to the structure of the vessel (Lusis 2000). Growth factors and cytokines released during atherosclerosis can cause a phenotypic change of VSMC from a contractile state to an active, synthetic state (McLaren et al. 2011). This allows the VSMCs to migrate, proliferate and produce ECM degrading enzymes (Newby and Zaltsman 1999). Growth promoters and inhibitors regulate the migratory activities of VSMC. Platelet-derived growth factor (PDGF), endothelin (ET)-1, thrombin, fibroblast growth factor (FGF), heparin-binding epidermal growth factor (HB-EGF), insulin-like growth factor (IGF-1), IFN-γ, and IL-1 have all been shown to promote migration of VSMC (Newby and Zaltsman 1999; Rudijanto 2007). The inhibitors of VSMC migration include NO and TGF-β (Rudijanto 2007). Within VSMCs intracellular signalling pathways are activated after the binding of extracellular ligands to the cell receptors, this activation promotes changes in gene regulation that lead to a migratory phenotype: some of the cytoplasmic and membrane-bound signaling proteins most associated with cell migration are Ras, Rho, cell division control protein 42 (Cdc42), focal adhesion kinase (FAK), phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK)s (Rudijanto 2007). VSMC migration involves a dominant plasma membrane leading edge, protruding to contact with an extracellular substrate to form focal adhesions (Rudijanto 2007). A cascade of intracellular signal transduction results in actin filament alignment and myosin contraction within the leading edge, this is followed by disengagement of focal adhesions over the remainder of the cell surface and contractile movement towards the leading edge (Rudijanto 2007). VSMC migration leads to an increased size of atherosclerotic lesion, but the lesion is usually more stable (Newby and Zaltsman 1999). Increasing the migration of VSMC during atherosclerosis could be a mechanism to produce more stable plaques that are less vulnerable to rupture.

1.1.6.2 – VSMC proliferation

VSMC proliferation and neo-intima formation are important events during atherosclerosis. Diverse signal transduction systems have been proposed that could regulate VSMC proliferation, among them nuclear factor κ-light-chain-enhancer of activated B cells (NFκ-B), MAPK and PI3K (Rudijanto 2007). A role of MAPKs in the induction of cellular proliferation has been described not only in VSMCs, but also in a variety of cell types and tissues after interaction of growth factors with their receptors (Rudijanto 2007). Several growth factors have been proposed to regulate VSMC proliferation through activation of pathways including PDGF, FGF, IGF-1 and TGF-β (Newby and Zaltsman 1999; Rudijanto 2007). Proliferation is not a quick process during atherosclerosis; usually very low rates of proliferation are observed in human plaques (0-1% proliferating cell nuclear antigen (PCNA) index). PCNA is synthesised during the cell cycle and is a useful marker of cell proliferation (Hsu et al. 1993). However, the long duration of the disease process (decades) means that plaques do increase in size and proliferation becomes an important regulatory factor of plaque stability (Newby and Zaltsman 1999).

1.1.6.3 – VSMC apoptosis

Apoptosis is a programmed form of cell death; extracellular signals activate signalling through cysteine and caspase proteases. The resultant signals cause apoptosis to be initiated (Newby 2006). Under normal physiological conditions human VSMCs within blood vessels show low apoptosis rates. During atherosclerosis additional factors such as inflammatory cytokines, T-cells, and modified-LDL are present that influence VSMC apoptosis (Rudijanto 2007). VSMC that were isolated from atherosclerotic plaques were intrinsically more sensitive to apoptosis, compared with cells from normal vessels (Clarke and Bennett 2006). One mechanism that could explain this observation is that VSMCs express death receptors, whose ligands are released and are present within the atherosclerotic plaque. Physiologically, combinations of cytokines such as IL-1β, IFN-γ, and TNF-α increase the expression of surface death receptors (Rudijanto 2007). Other proposed mechanisms of increasing apoptosis levels include inflammation, oxidative stress, integrin adhesions and modified-LDL (Bennett 1999). VSMC apoptosis during atherosclerosis reduces the stability of the plaque by lowering the number of VSMC

residing within the fibrous cap. This causes a weakening of the cap and reduces the net amount of ECM matrix components that can be released to maintain the stability (McLaren et al. 2011). During an *in vivo* study, inducible selective VSMC apoptosis accelerated atherosclerosis by promoting stenosis, medial degradation and decreasing expansive remodeling (Clarke et al. 2008). Understanding the role of VSMC apoptosis in plaque stability could lead to specific apoptosis inhibitors that target cells only within the fibrous cap of the lesion.

1.1.6.4 – ECM synthesis

During the formation and the maintenance of the fibrous cap the VSMC are the main producers of ECM matrix components that provide the strength and stability to the cap (Doran et al. 2008). Whereas most of the ECM within a healthy artery is type I and type III fibrillar collagen, atherosclerotic lesions tend to contain VSMC that produce mostly proteoglycans (Doran et al. 2008). These proteoglycans within the developing plaque can bind to LDL allowing it to be quickly oxidised, enhancing lipid uptake by macrophages. The increased amount of oxidated-LDL stimulates VSMC to secrete larger and more highly sulfated proteoglycans, which amplifies this action leading to faster lesion progression (Doran et al. 2008). The constant production of ECM matrix components is also very important in ECM turnover. Pro-inflammatory cytokines such as IFN-γ have been shown to reduce collagen I and III synthesis from VSMC, therefore IFN-γ stimulation could lead to impaired ability of VSMCs to repair and maintain the mechanical integrity of the plaque (Libby and Aikawa 2002). This observation has been confirmed in mouse models of atherosclerosis: reduction of the expression of the IFN-γ receptor led to collagen accumulation within the plaques and a more stable phenotype (Gupta et al. 1997). The reduction of ECM component synthesis from VSMC along with the increase in VSMC apoptosis (section 1.1.6.4), could act synergistically to promote the instability of the fibrous cap, rendering the plaque vulnerable to rupture. The involvement of pro-inflammatory cytokines in this process usually means that the plaque becomes weaker where there are large numbers of macrophages, which is usually in the shoulder regions of the plaque.

1.1.6.5 – Plaque Rupture

It has been recognised during clinical observations that acute symptoms such as heart attack and stroke usually do not occur due to the plaque critically narrowing the artery: the symptoms occur when the plaque ruptures and a thrombotic reaction is initiated (Lusis 2000). The thrombotic reaction is initiated because macrophage foam cells show induced pro-coagulant tissue-factor in the core of atherosclerotic plaques (Libby and Aikawa 2002). When the tissue-factor comes into contact with blood, platelets quickly cause thrombosis, therefore giving rise to the clinical symptoms that are observed (Libby and Aikawa 2002). *In vitro* studies have shown that CD40 ligand, a product of T-cell activation, augments tissue-factor expression considerably more than conventional pro-inflammatory cytokines (Mach et al. 1997a). Taking these observations into consideration, the stability of the mature atherosclerotic plaque is very important in controlling acute events (Halvorsen et al. 2008). There are many factors within the plaque that can alter stability, and therefore trying to study and understand these factors is very important in the quest for plaque stabilising therapies. Some of the factors that do influence stability are cytokine production, protease expression, VSMC proliferation, VSMC migration and VSMC apoptosis (Newby 2006). Vulnerable atherosclerotic plaques usually have a thin fibrous cap surrounding a large necrotic core. Within the vulnerable plaque there is a high level of inflammation that causes high levels of pro-inflammatory cytokines to be released (Halvorsen et al. 2008). The cytokines are regulated and produced as a product of T-cell activation during disease progression (Mallat et al. 2009; Taleb et al. 2008). The general characteristics of stable and unstable plaques are outlined in Table 1.3.

Table 1.3: Characteristics of stable and unstable plaques (Halvorsen et al. 2008)

Features	Vulnerable Plaque	Stable Plaque
Fibrous Cap	-	+
Lipid-rich core	+	-
Inflammation	+	-
Neovascularisation	+	-
Calcification	-	+
Matrix remodelling	+	-
Necrosis	+	-
Apoptosis	+/-	+/-

1.1.7 – MMP proteases and plaque stability

One of the major families of proteases that are involved in remodelling the fibrous cap during atherosclerosis is the MMPs. The mammalian family of MMPs are a group of 23 enzymes that are able to degrade ECM components; they are usually secreted as an inactive pro-form, although some are matrix bound (MMP-14, -17, -25 and -26) (Newby 2012). Combinations of MMPs have the ability to extensively degrade the ECM and many of the MMPs can activate other family members; this allows MMPs to act in a protein cascade therefore amplifying their responses (Newby 2012). The members of the MMP family and their substrates are shown in Table 1.4. These proteases have been extensively studied in the context of atherosclerotic plaque stability (Newby 2005; Newby 2007, 2012; Newby et al. 2009). The MMPs are capable of degrading collagen, proteoglycans and elastin, which are all major constituents of the fibrous cap ECM (Halvorsen et al. 2008). MMPs expression was observed within atherosclerotic plaques from macrophages, VSMC and endothelial cells (Newby 2006; Nikkari et al. 1995). Pro-inflammatory cytokines also regulate the expression of MMPs; for example, IL-1, IL-4 and TNF-α increase the expression of a wide range of MMPs in VSMC (Bond et al. 2001; Moon

et al. 2004; Newby 2006). The effect of the pro-inflammatory cytokines are synergistic with growth factors such as, PDGF and FGF-2 (Newby 2006). Another regulator of MMP action is the presence of a group of MMP inhibitors, the tissue inhibitor of metalloproteinases (TIMP). The balance between MMP/TIMP is very important to what effect the MMPs have on atherogenesis (Newby 2012). When there is high stretch or an injury to the endothelium, the balance is pushed towards higher MMP activity and less TIMPs (Newby 2006). MMPs have been shown to degrade the matrix during plaque destabilisation and rupture, which implicates a pathogenic role for the proteases (Newby 2006). The MMPs can act on platelets in the blood to prime them for aggregation, another suggested pro-athogenic role (Choi et al. 2008). Having said this, a dual role for the MMPs has been suggested because the proteases can promote VSMC migration, therefore increasing the stability of the plaque (Newby 2005). The actions of MMPs have also been implicated in the regulation of apoptosis within atherosclerotic plaques (Newby 2006). The different isoforms of the MMPs can have a different profile of substrates, assigning them different regulatory actions on atherogenesis (Newby 2012). The interesting isoform specific regulatory actions that the MMPs exhibit make them very interesting to review. The mechanism by which the isoforms regulate VSMC migration, proliferation and apoptosis will be outlined in detail in the following sections.

Table 1.4: MMPs and their matrix substrates (Newby 2006)

MMP classification	Substrates
MMP-1	Collagen I, II, III, VII, VIII, X and gelatin, aggrecan, casein, nidogen, serpins, versican, perlecan, proteoglycan link protein, tenascin-C
MMP-2	Collagen I, IV, V, VII, X, XI, XIV and gelatin, aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan link protein and versican
MMP-3	Collagen II, IV, IX, X and gelatin, aggrecan, casein, decorin, elastin, fibronectin, laminin, nidogen, perlecan, proteoglycan link protein and versican
MMP-7	Collagen I, II, III, IV, V, X and aggrecan, casein, elastin, enactin, laminin and proteoglycan link protein
MMP-8	Collagen I, II, III, V, VII, VIII, X and gelatin, aggrecan, laminin and nidogen
MMP-9	Collagen IV, V, VII, X, XIV and fibronectin, laminin, nidogen, proteoglycan link protein and versican
MMP-10	Collagen III, IV, V and gelatin, fibronectin, laminin and nidogen
MMP-11	Laminin
MMP-12	Elastin
MMP-13	Collagen I, II, III, IV, V, IX, X, XI and gelatin, aggrecan, fibronectin, laminin, perlecan, tenascin
MMP-14	Collagen I, II, III and gelatin, aggrecan, dermatan sulphate proteoglycan, fibrin, fibronectin, laminin, nidogen, perlecan, tenascin and vitronectin
MMP-15	Collagen I, II, III and gelatin, aggrecan, fibronectin, laminin, nidogen, perlecan, tenascin, vitronectin
MMP-16	Collagen I, III and gelatin, aggrecan, casein, fibronectin, laminin, perlecan and vitronectin
MMP-17	Gelatin, fibrin, fibronectin

1.1.7.1 – MMPs and VSMC migration

The mechanism by which MMPs act to promote VSMC migration could happen in a multitude of different ways. In the first instance VSMC cells need to be cleaved free from their basement membrane (BM) attachment within the medial layer of the vessel before migration (Newby 2006; Pauly et al. 1994). During an organ culture experiment, all migrating VSMC lacked BMs suggesting the BM attachment had been cleaved when they migrated away from the media (Aguilera et al. 2003). Furthermore a role for MMPs was implicated in the cleavage of attachment to the BM: addition of TIMP activity by gene transfer led to preserved medial BM attachment and inhibited VSMC migration from the media (Aguilera et al. 2003). When VSMCs are actually migrating many signalling pathways are activated that govern the action of re-organising the cytoskeleton, forming reversible contacts with ECM components and integrin binding (Newby 2006). Hence the action of MMPs, by degrading BMs can indirectly facilitate a host of new ECM-integrin interactions leading to activation of focal adhesion kinase and increased migration (Koshman et al. 2011; Newby 2006; Owens et al. 2001). Cleavage of ECM components could also create cryptic integrin binding sites allowing for improved migratory behaviour of VSMCs. PDGF stimulated VSMC migrated more rapidly on cleaved collagen I in comparison to intact collagen I (Stringa et al. 2000). MMPs have also been shown to be able to cleave N-cadherin cell-cell interactions freeing up VSMC to migrate (Dwivedi et al. 2009; Newby 2006; Williams et al. 2010a). These findings provide evidence that the action of MMPs could act to increase VSMC migration during atherosclerosis. However, the action of MMPs on migration does seem to be specific to the isoform involved. Over-expression of MMP-14 and MMP-16 caused FAK cleavage reducing signalling through this pathway (Shofuda et al. 2004). This implies that specific MMPs could act to actually impair migration of VSMC (Newby 2006). These studies highlighted the need to produce specific protocols that inhibit the activity of specific MMPs within atherosclerosis models (Newby 2012).

1.1.7.2 – MMPs and VSMC proliferation

MMP activity has been suggested to potentially influence the proliferation of VSMC once they have migrated into the fibrous cap. The action of MMPs on N-cadherin cell-cell adhesions has been hypothesised to increase VSMC proliferation rates (Uglow et al.

2003). MMP action has also been suggested to increase the availability of membrane bound growth factors such as FGF-1 and -2 to VSMC (Newby 2006). Further studies looking into the mechanism of this action have produced conflicting and inconclusive results suggesting that other processes may govern proliferation, such as the action of serine proteases (Newby 2006). Improved experimental models to study the effect of MMP action on VSMC proliferation are required so that specific MMP actions can be elucidated and potentially inhibited to try and regulate the stability of atherosclerotic plaques through VSMC proliferation.

1.1.7.3 – MMPs and VSMC apoptosis

Another important role that MMPs have in atherosclerosis is controlling apoptosis of VSMCs. Apoptosis could be a very important factor in plaque stability as a reduction in VSMC numbers in the fibrous cap could lead to a less stable lesion, more vulnerable to rupture. There is potential for the MMPs to have both stimulatory and inhibitory actions on VSMC apoptosis. One mechanism by which MMPs could regulate apoptosis of VSMC is by cleavage of membrane bound survival factors (Newby 2006). MMP-2, -7 and -9 can cleave pro-HB-EGF and liberate soluble active growth factor (Cheng et al. 2007). Also, MMP-1, -2, -8 and -9 can cleave members of the IGF binding protein family therefore increasing the availability of IGF-1 (Fowlkes et al. 1994; Fowlkes et al. 1995; Newby 2006). These increases in active survival factors to VSMC suggest that specific MMPs can have a role in helping VSMC survive within atherosclerotic plaques. FAK activation triggered by MMP actions and ECM-integrin binding leads to cell survival signals by induction of p53 signalling pathway (Ilić et al. 1998). Specific, regulated MMP action helps activate FAK activity and could have a protective role against apoptosis (Newby 2006). However, excessive MMP activity can degrade ECM proteins and integrins leading to increased apoptosis and anoikis: anoikis is a form of programmed cell death initiated by loss of attachment to surrounding ECM components (Levkau et al. 2002). Cleavage of N-cadherin cell-cell adhesions by MMPs can also result in a loss of the survival signals that the adhesions provide (Uglow et al. 2003). Another mechanism by which MMPs could regulate apoptosis is by the cleavage of death ligands. MMP-7 has been shown to cleave pro-TNF-α, Fas and Fas-L in atherosclerotic plaques (Newby 2006). TIMP-3 has also been shown to be a stimulatory agent for apoptosis in VSMC. The mechanism by which this

occurs is poorly understood (Newby 2006). The regulation of apoptosis by specific MMPs and the knowledge about the outcome of these specific actions are relatively incomplete. There is a large scope for research investigating how inhibiting or stimulating specific MMP activities could lead to regulation of apoptosis leading to a more stable atherosclerotic plaque (Newby 2012).

1.1.7.4 – MMP inhibition and potential novel therapeutics for atherosclerosis

The dual role and actions of the MMPs make them a difficult group of enzymes to profile in detail. It is important that the individual MMPs acting to stabilise the plaque are identified, as they offer a potential therapeutic opportunity in the disease (Newby 2008). Some specific MMP inhibitors have been studied within apoE null mouse models of atherosclerosis. The findings from the previous studies have shown that MMP-2, -3 and -9 can enhance plaque stability by promoting intima formation (Johnson et al. 2005; Newby 2012). MMP-7, -8, -12, -13 and -14 can increase ECM destruction, inflammation and apoptosis leading to a reduction in plaque stability (Johnson et al. 2005; Johnson et al. 2008; Newby 2012). These findings provide insight into potential therapies that could inhibit MMPs that cause a reduction in plaque stability and therefore increase the likelihood of clinical consequences (Newby 2012). Two studies have recently used MMP inhibition in apoE null mice. Firstly a MMP-12 inhibitor called RXP470.1 was administered to apoE null mice. RXP470.1 arrested plaque enlargement, improved the ratio of VSMC to macrophages and decreased lipid core formation, macrophage apoptosis and media elastin breaks (Johnson et al. 2011). Secondly, a highly selective inhibitor of MMP-13 called MMP13i-A was administered to apoE null mice. The result was inhibition of collagenolysis and preserved collagen levels within plaques (Newby 2012; Quillard et al. 2011). The results of these studies in mice are promising. However, the expression profiles of MMPs from mouse and human plaques are very different. Before MMP inhibition can be used as a major clinical treatment individual MMP profiles need to be very well understood in human plaques. This would allow inhibition of specific MMPs in pre-clinical and clinical trials (Newby 2012).

1.1.7.5 – Other proteases active within the plaque

The MMPs have been extensively studied during the progression of atherosclerosis. They have a large array of regulatory actions over important cellular events during the disease. This has led researchers to ask, do any other proteases act within the plaque in a similar manner?

1.2 - ADAMTS proteases

1.2.1 – ADAMTS Background

ADAMTS proteases are a family of 19 proteins that share a similar domain pattern and substrate range; they are structurally related to the a disintegrin and metalloproteinase (ADAM) and MMP protease families and have been implicated in a number of pathophysiological conditions (Porter et al. 2005; Salter et al. 2010; Tortorella et al. 2009). The founding member of the ADAMTS proteases was first cloned, identified and named in a study carried out in 1997 (Kuno et al. 1997), from here the family has grown to 19 members (Porter et al. 2005; Salter et al. 2010). ADAMTS proteases are non-membrane bound proteins that act on a wide variety of ECM substrates including pro-collagen, proteoglycans, hyalectans and cartilage oligomeric matrix protein (COMP) (Jones and Riley 2005). The physiological roles of the ADAMTS proteases are now beginning to be understood; a role in cartilage turnover and also in atherosclerosis has been suggested (Salter et al. 2010; Tortorella et al. 2009). The gene names, protein names, chromosome location and known substrates for the ADAMTS enzymes are summarised in Table 1.5.

Table 1.5: ADAMTS proteases and their known substrates (Salter et al. 2010)

Protein Name	Alternative Names	Chromosome Location	Known Substrates
ADAMTS-1	METH-1, aggrecanase-3	21q21	Aggrecan, Versican
ADAMTS-2	PCINP	5q35	Type I, II, III pro-collagens
ADAMTS-3	KIAA0366	4q21	Type II pro-collagen
ADAMTS-4	Aggrecanase-1, KIAA0688	1q23	Aggrecan, brevican, COMP, fibromodulin, decorin, biglycan, versican
ADAMTS-5	Aggrecanase-2, ADAMTS-11	21q21	Aggrecan, biglycan, fibromodulin, decorin, brevican, versican
ADAMTS-6		5q12	
ADAMTS-7		15q24	
ADAMTS-8	METH-2	11q25	Aggrecan
ADAMTS-9	KIAA1312	3p14	Aggrecan, versican
ADAMTS-10		19p13	
ADAMTS-12		5q35	
ADAMTS-13	vWFCP	9q34	Von willebrand factor
ADAMTS-14		10q21	Type I pro-collagen
ADAMTS-15		11q25	Aggrecan
ADAMTS-16		5p15	
ADAMTS-17		15q24	
ADAMTS-18		16q23	
ADAMTS-19		5q31	
ADAMTS-20		12q12	Versican

The ADAMTS proteases are closely related to the ADAM proteins that are involved in activation of cell surface molecules and growth factors. However the ADAM family is made up of transmembrane proteins, whereas, the ADAMTS group are secreted enzymes (Tang and Hong 1999). The ADAMTS enzymes have also been shown to be able to bind to ECM components, possibly through interactions with various glycosaminoglycans (GAGS).

Members of the ADAMTS family possess structural homology and the archetypal structure can be subcategorised into different domains, modules and motifs (Salter et al. 2010). The family share many homologous protein modules and domains, including a metalloproteinase domain, a disintegrin domain, a cysteine-rich domain and an EGF repeat (Tang and Hong 1999). Due to the many domains within the ADAMTS genomic structure, there is considerable scope for the generation of multiple isoforms of the genes by alternative splicing. All of the domain structures of the ADAMTS family are shown in Figure 1.4.

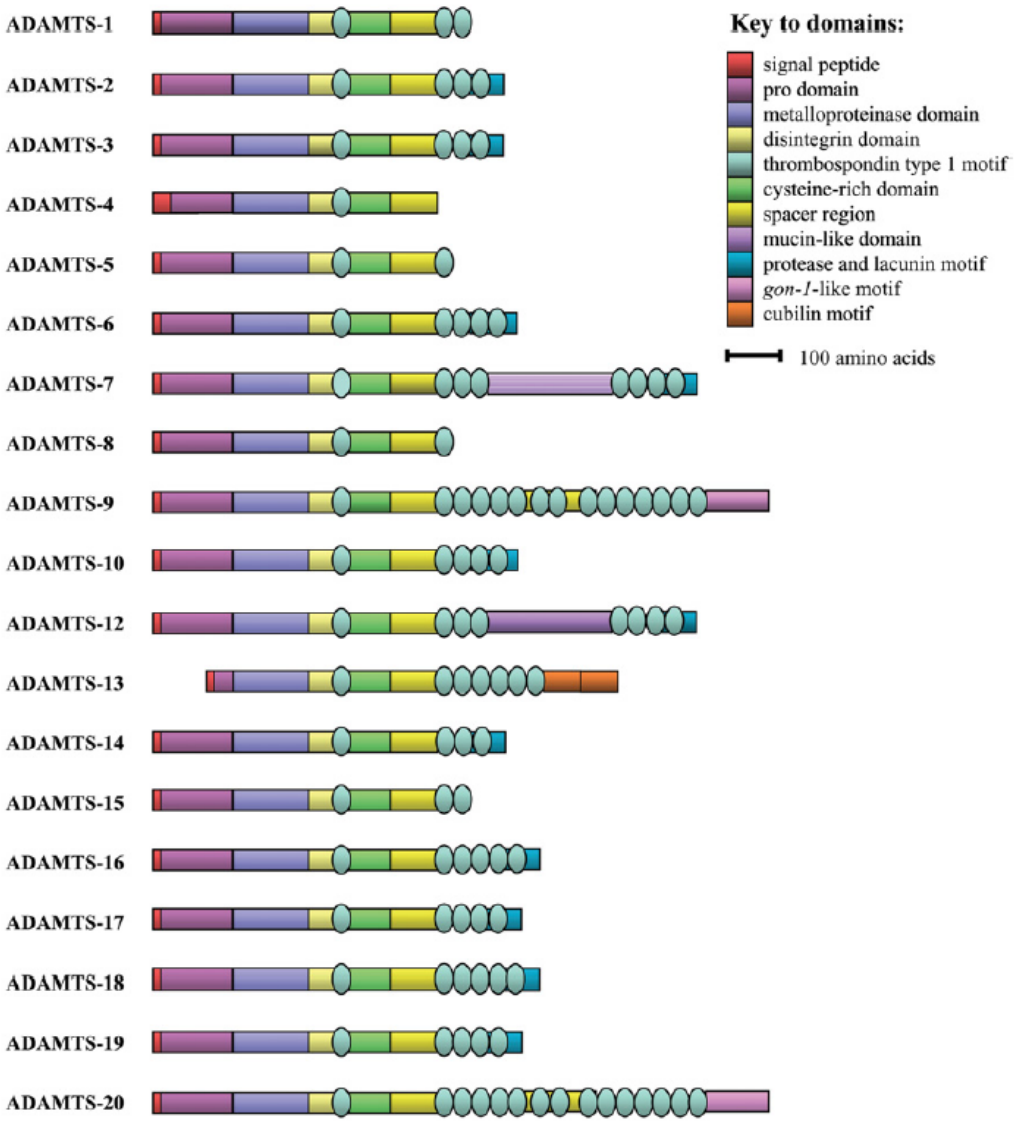


Figure 1.4: **Domain structure of the ADAMTS proteinases (Porter et al. 2005).**

All of the ADAMTS enzymes are initially synthesised in an inactive form with a pro-domain near their N terminus. The name of the inactive form of the ADAMTS enzymes is a pre-proenzyme. In order for the ADAMTS enzymes to perform their biological function, they require activation by cleavage of the pro-domain (Stanton et al. 2011). The ADAMTS proteases undergo N-terminal processing by a signal peptidase during translation and trafficking of the protein. Then the pro-domain is removed, creating the final active enzyme for secretion. The pro-domain has been shown to be important for enzyme latency and also in the correct folding and secretion of the protein (Porter et al. 2005).

The C-terminal domains of the ADAMTS enzymes have a large amount of control over the substrate specificity and localisation of the protease. The different domains that are possessed by the individual family members control what substrates they can act upon, and where they are localised within the extracellular matrix (Stanton et al. 2011). Another factor that gives variation to the C-terminal structure is C-terminal processing (Porter et al. 2005). This has been shown to occur on a few of the family members, but not all. Two of the family members that exhibit C-terminal processing are shown in Figure 1.5, along with the cleavage points.

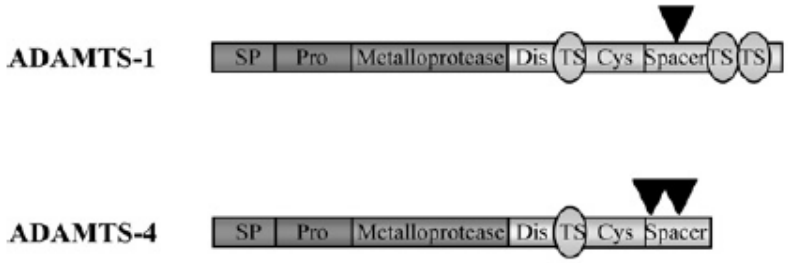


Figure 1.5: C-terminal processing showing the cleavage points for ADAMTS-1 and -4 (Porter et al. 2005).

The ADAMTS protease family is sub-divided into four classes based on structural and functional similarities, the classes are named the proteoglycanases, the procollagen-n-peptidases, von Willebrand cleaving factor and those ADAMTS proteases whose function remains to be fully elucidated (Salter et al. 2010). The proteoglycanases are the sub-class that is most relevant to atherosclerosis. The proteoglycanases ADAMTS-1, -4, -5, -8, -9

and -15 possess anti-angiogenic activity, and out of these ADAMTS-1, -4, -5 and -9 are able to cleave aggrecan (Cross et al. 2005). Most importantly in relation to their activity during atherosclerosis ADAMTS-1, -4, -5 and -9 are also able to cleave versican (Cal et al. 2002; Cross et al. 2005; Sandy et al. 2001; Westling et al. 2004).

The ADAMTS enzymes are regulated by various mechanisms, including transcriptional control, TIMPs, and also activating conditions. TIMPs are inhibitors of the MMPs and they do have a variable effect on the members of the ADAMTS family. Different members of the ADAMTS family have different inhibition profiles. For example ADAMTS-4 and -5 are both inhibited by TIMP-3, although they are insensitive to TIMP-1, -2, and -4 (Porter et al. 2005). Another mechanism of regulation for ADAMTS enzymes is transcriptional control. The exact mechanisms of transcriptional control are still poorly understood, although it has been shown that many of the ADAMTS family members are regulated by growth factors, hormones, and inflammatory cytokines (Porter et al. 2005).

1.2.2 - ADAMTS proteases in atherosclerosis

MMPs have been suggested to be major regulators of the atherosclerotic process through re-modelling of the plaque ECM (Newby et al. 2009). As the protein families are structurally related, the role for ADAMTS proteins in atherosclerosis could be similar to that of the MMPs (Salter et al. 2010). ADAMTS-1, -4, -5 and -8 are expressed within human atherosclerotic plaques, and macrophages have been identified as the major contributors towards ADAMTS expression in the disease (Jonsson-Rylander et al. 2005; Lee et al. 2011; Wagsater et al. 2008). ADAMTS proteases are also expressed in VSMC and endothelial cells, but to a lower extent than macrophages and foam cells (Jonsson-Rylander et al. 2005; Wagsater et al. 2008). ADAMTS-1 expression has been studied in various wild type mouse tissues and has been shown to be at the highest level in the aorta (Jonsson-Rylander et al. 2005). ADAMTS-4 mRNA is present in $LDLR^{-/-}ApoB^{100/100}$ aortas before any atherosclerotic lesions are visible and the level of expression increased as the lesions become more advanced (Wagsater et al. 2008).

The central hypothesised role of the ADAMTS proteases within the atherosclerotic plaque is cleavage of versican potentially leading to regulation of migration, proliferation, apoptosis and other cellular events within VSMC and macrophages (Salter et al. 2010). It has been shown previously that some ADAMTS enzymes are aggrecanases that are able to cleave the protein versican (Table 1.5), including ADAMTS-1, -4 and -5 (Salter et al. 2010). Versican is a large ECM proteoglycan that is highly expressed in atherosclerotic plaques (Wight 2002). The highly interactive nature of the molecule gives a basis for its importance in disease progression (Wight 2002). The molecule is able to interact with cell surface molecules and regulate cell adhesion and survival, cell proliferation, cell migration and ECM assembly (Wight 2002). All of these regulatory properties of versican are important in atherosclerosis therefore members of the ADAMTS family could cleave the proteoglycan and play a major regulatory role (Wight 2002). Although the exact role of versican cleavage in atherosclerosis is yet to be deciphered, versican degradation has been implicated in decreasing plaque stability at the later stages of atherosclerosis (Wagsater et al. 2008).

The role of versican cleavage within atherosclerosis is a complicated one; expression has been detected within all three arterial wall layers and is interestingly up-regulated during all forms of vascular disease (Lemire et al. 2007). Versican is likely to provide structure and strength to the vasculature through its interaction with hyaluronan (Wight 2002). Versican-hyaluronan complexes are necessary for the migration and proliferation of VSMCs following wounding and also serve as an attachment for macrophages and lymphocytes, suggesting that the complexes may influence the retention of inflammatory cells (Evanko et al. 1999). The chondroitin sulphate chains of versican can interact with chemokines and adhesion molecules that could influence recruitment and migration of vascular cells including macrophages (Hirose et al. 2001; Kawashima et al. 2000). The chondroitin sulphate chains also interact with LDL particles in advanced lesions leading to increased LDL uptake in both VSMCs and macrophages (Llorente-Cortés et al. 2002). These findings suggest that cleavage of versican and other ECM proteoglycans by ADAMTS proteases during atherosclerosis could have regulatory control over important cellular aspects of the disease. The possible roles that the ADAMTS proteases could play during atherosclerosis are outlined in Figure 1.6.

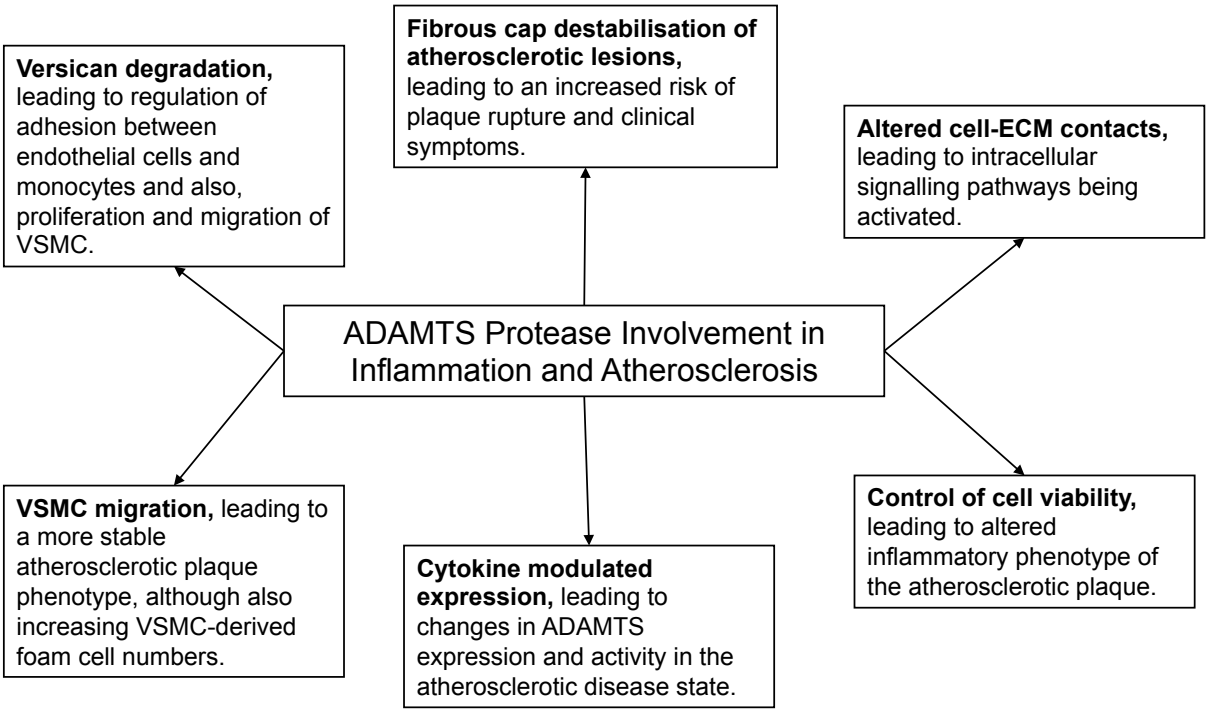


Figure 1.6: Possible regulatory roles of the ADAMTS proteases during atherosclerosis (Salter et al. 2010).

1.3 – Inflammation and atherosclerosis

1.3.1 – Cytokine action during atherosclerosis

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

It has been shown that atherosclerosis is a state of chronic inflammation within the vascular wall (Lusis 2000). A large range of cytokines are released at different stages of

the disease and these have been studied and reviewed in relation to them being pro- or anti-atherogenic (Tedgui and Mallat 2006). A selection of important regulatory cytokines during atherosclerosis is outlined in Table 1.6.

Table 1.6: Important regulatory cytokines during atherosclerosis (McLaren et al. 2011; Tedgui and Mallat 2006)

Pro-atherogenic	Anti-atherogenic
IFN-γ	TGF-β
IL-1β	IL-10
TNF-α	IL-33
Macrophage migration inhibitory factor-1 (MIF)	IL-17A
TNF-like weak inducer of apoptosis (TWEAK)	IL-6
TNF-like protein 1A (TL1A)	
IL-17A	
IL-6	

1.3.1.1 – Cytokine regulation of foam cell formation

It has been observed from previous studies that foam cell formation is regulated by various cytokines; they can act to either increase or decrease the level of modified LDL retention (McLaren et al. 2011). Foam cell formation is a critical pathological step during the progression of the atherosclerotic plaque. The classical pro-inflammatory cytokines IFN-γ, IL-1β and TNF-α increase the level of foam cell formation during atherosclerosis, along with other cytokines such as TWEAK, TL1A and MIF (McLaren et al. 2010a; McLaren et al. 2011; Moreno et al. 2009). The classical anti-inflammatory cytokine TGF-β has been shown to reduce the level of foam cell formation during atherosclerosis, along with IL-10 and IL-33 (Draude and Lorenz 2000; McLaren et al. 2011; McLaren et al. 2010c; Michael et al. 2012).

1.3.1.3 – Cytokine regulation of ADAMTS expression

The regulation of critical steps such as foam cell formation by cytokines highlights the importance of inflammatory mediators during plaque maturation and development. The regulation of ADAMTS gene transcription by cytokine action has been studied, but not in

any major detail. The following section will review the previous literature that has investigated cytokine regulation of the ADAMTS proteases. The numerous cytokines that are present within the atherosclerotic plaque could potentially give rise to cause numerous changes in transcriptional gene regulation affecting the course of the disease (Tedgui and Mallat 2006). The understanding of these potential regulatory mechanisms would give the researcher a better idea of the role that ADAMTS proteases have during atherosclerosis progression.

More research has been carried out to ascertain the regulation of ADAMTS-4 and -5 expressions in human chondrocytes during osteoarthritis (OA) than that for atherosclerosis (Salter et al. 2010). ADAMTS-4 and -5 are implicated in the breakdown of cartilage ECM during OA and the regulation of their activity by pro-inflammatory cytokines has been investigated; the pro-inflammatory cytokines IL-1β and TNF-α have been shown to increase the expression of ADAMTS-4 and -5 in chondrocytes (Sylvester et al. 2011; Wang et al. 2011). It has also been observed that IL-6 can positively regulate the expression of ADAMTS-4 in fibroblast-like synoviocytes in patients suffering from rheumatoid arthritis (RA) (Mimata et al. 2012).

In vitro studies have been carried out that have investigated the regulation of ADAMTS proteases in macrophages by cytokines in relation to atherosclerosis. The first study was carried out in 2008 and aimed to study the expression and regulation of ADAMTS proteases in macrophages, before and after differentiation, by IFN-γ, IL-1β and TNF-α (Wagsater et al. 2008). ADAMTS-4 and -8 were induced upon monocyte to macrophage differentiation and macrophage expressions of ADAMTS-4, -7, -8 and -9 mRNA were further enhanced upon stimulation with IFN-γ or TNF-α. On the other hand, IFN-γ attenuated the expression of ADAMTS-1 (Wagsater et al. 2008). The second study was carried out in 2011 and looked at the effect of TGF-β stimulation on ADAMTS-4 expression in macrophages (Salter et al. 2011). This study showed that the anti-atherogenic cytokine TGF-β inhibited the expression of ADAMTS-4 in human macrophages and siRNA-mediated knockdown revealed a critical role for Smads, p38 mitogen-activated protein kinase and c-Jun in the action of TGF-β on ADAMTS-4 mRNA expression (Salter et al. 2011). These findings suggest potentially important roles for

ADAMTS proteases during atherosclerosis and show how regulation by specific cytokines can influence their expression.

1.3.2 – Cytokines used within this project

1.3.2.1 - TGF-β

The TGF-β superfamily has been shown to contain over 35 members that are structurally related, and share similar biological actions; the family consists of the prototypic member TGF-β and a range of related factors including activins, nodals and bone morphogenic proteins. The mature active forms of the family are normally composed of dimers between two 12-15kDa subunits (Singh and Ramji 2006a). The cytokine TGF-β has three isoforms that show around 70-80% sequence homology to each other and exhibit similar biological actions, and so have been assigned the generic term TGF-β (Singh and Ramji 2006a). TGF-β is synthesised as a pro-protein (pro-TGF-β) that is homo-dimeric and has a molecular weight of 75kDa. The dimeric pro-protein is cleaved within the golgi apparatus to form the mature 24kDa dimer (Ruiz-Ortega et al. 2007). The mature TGF-β dimer has affinity for other dimers of TGF-β; therefore small latent complexes are usually secreted. In the latent complex form, the TGF-β is unable to bind to its receptor (Ruiz-Ortega et al. 2007). Another protein called latent TGF-β binding protein (LTBP) can also bind to the small latent complexes, further inhibiting TGF-β from being physiologically active. Due to the latent behaviour of the complex formation, the dissociation of TGF-β from the complex is an extremely important step in activating the biological activity of the cytokine (Singh and Ramji 2006a). The dissociation of the TGF-β from its complex is initiated by thrombospondin-1, plasmin, cathesin D, furin-like pro-protein convertases, integrins, heat treatment, changes in pH, and a number of other agents (Bailly et al. 1997; Singh and Ramji 2006a). TGF-β is expressed in many cell types that relate to atherosclerosis in the vascular wall, these include EC, VSMC, monocytes/macrophages, platelets and T-cells (Singh and Ramji 2006a).

TGF-β mainly exerts its biological effect through the Smad signal transduction pathway, although the MAPK pathway has also been implicated (Salter et al. 2011; Singh and Ramji

2006a). The Smad signalling pathway is outlined in Figure 1.7. There are two main types of receptor on which TGF-β can bind; they are called TGF-βRI and TGF-βRII (Singh and Ramji 2006a). They are transmembrane serine/threonine kinase receptors that are able to activate downstream events within the cell. Another TGF-β receptor exists that has no intrinsic kinase activity and its function is not fully understood, which is called TGF-βRIII (Ruiz-Ortega et al. 2007). The signal transduction cascade is initiated because TGF-β has a strong affinity to TGF-βRII, to which the cytokine binds and this causes TGF-βRI to be recruited into a heteromeric complex. TGF-βRII is then able to phosphorylate TGF-βRI and cause a conformational change that allows the Smad family of signal transducers to be activated by phosphorylation (Singh and Ramji 2006a). When activated and aided by the Smad anchor for receptor activation (SARA), R-Smads (Smad-2 and -3 for TGF-β) and Co-Smads (Smad4) are able to form a complex that enters the nucleus and acts as a transcription factor for TGF-β regulated genes. The activated Smad complex is also able to affect the activity of other transcription factors by protein-protein interactions. Another family of Smad exists, which are called I-Smads, these are able to inhibit the formation of the Smad complex and therefore inhibit the effect of TGF-β within the cell (Singh and Ramji 2006a).

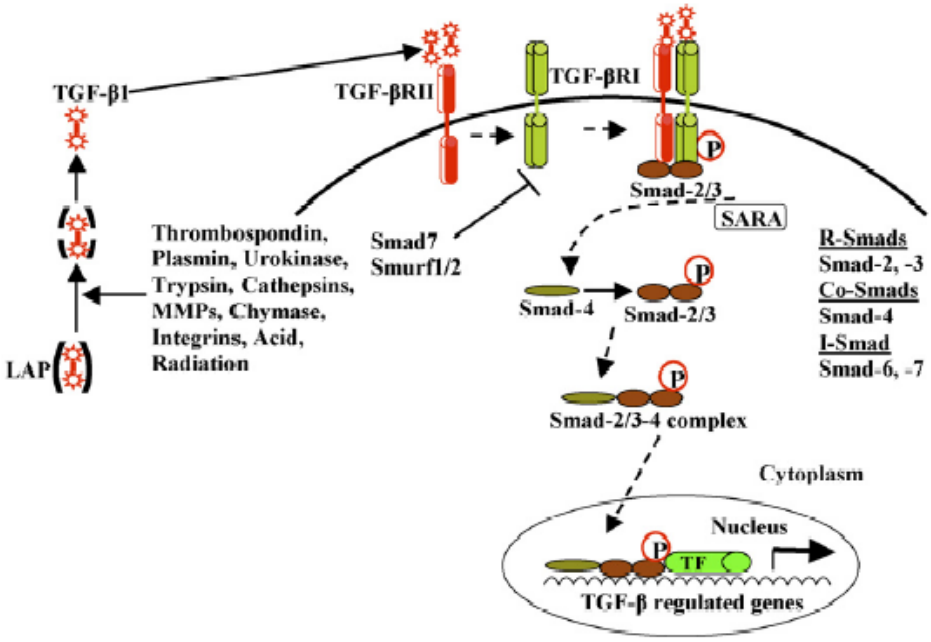


Figure 1.7: The receptors and Smad signal transduction cascade following TGF-β binding (Singh and Ramji 2006a).

TGF-β is secreted as a latent complex and is activated by various factors. The active cytokine can then interact with the receptors. TGF-βRI and TGF-βRII form a complex when TGF-β binds causing the Smads to be activated. A complex between R-Smads and Co-Smads is able to enter the nucleus and act as a transcription factor.

TGF-β is highly expressed in atherosclerotic plaques and plays many important roles in vascular fibrosis (Singh and Ramji 2006a). As TGF-β is highly expressed in atherosclerotic lesions exhibiting a range of biological effects, it has a profile of different effects on a range of cell types during plaque formation (Ruiz-Ortega et al. 2007). TGF-β predominantly shows anti-atherogenic properties, highlighted by low serum levels being observed in patients with advanced atherosclerosis and regions of the aorta with a high probability of lesion development displaying low levels of TGF-β expression (Singh and Ramji 2006a). The inhibition of TGF-β activity and/or expression in mouse models of atherosclerosis results in accelerated lesion development and an elevated inflammatory response (Michael et al. 2012). TGF-β increases the production of IL-10 that is an anti-inflammatory cytokine which exhibits anti-atherogenic actions (Ruiz-Ortega et al. 2007). TGF-β also reduces the uptake of modified-LDL in a Smad dependent manner, through

regulation of key uptake and efflux genes from macrophages (Michael et al. 2012; Singh and Ramji 2006b). TGF-β can reduce foam cell formation by attenuating the expression of the scavenger receptors responsible for taking up modified LDL (Michael et al. 2012). TGF-β also has regulatory actions over the stability of the plaque (Lutgens and Daemen 2001). It increases the expression of ECM components such as fibronectin, collagen and plasminogen activator inhibitor-1 (PAI-1), it also decreases the production of collagenases and induces TIMP expression (Ruiz-Ortega et al. 2007). TGF-β also has an effect on VSMC migration and proliferation, at high concentrations only the migration and proliferation of VSMCs is reduced. This suggests that there must be a concentration dependent regulation over VSMC migration and proliferation by TGF-β (Ruiz-Ortega et al. 2007). TGF-β has been shown to decrease the initiation of lesions by acting upon ECs, reducing the expression of adhesion proteins, such as the selectins and ICAM-1 (Lutgens and Daemen 2001). These effects on endothelial cells, along with the other actions presented here, outline that TGF-β has an anti-atherogenic role to play in almost all stages of disease progression.

1.3.2.2 - IFN-γ

IFN-γ is a type II member of the IFN family of cytokines and is produced by many cell types involved in the immune reaction (McLaren and Ramji 2009). The cell types that express IFN-γ include T-lymphocytes, natural killer cells, macrophages, dendritic cells, and B-lymphocytes (McLaren and Ramji 2009). IFN-γ has a wide range of actions and exerts its biological effects by activating various signalling pathways (McLaren and Ramji 2009). The major action of IFN-γ is transduced through the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, however the pattern of activation is complex (van Boxel-Dezaire and Stark 2007). Some studies have shown that alternative pathways are activated such as the MAPKs and PI3Ks, along with differential activation of the STAT proteins in different cell types (Smyth et al. 2012; van Boxel-Dezaire and Stark 2007). IFN-γ activity is observed after binding to the cellular receptor that is made up of two subunits named IFN-γR1 and IFN-γR2. IFN-γ binding causes the dimerisation of the receptor subunits to create an IFN-γR receptor complex (McLaren and Ramji 2009). The mechanism of IFN-γ signalling is outlined in Figure 1.8.

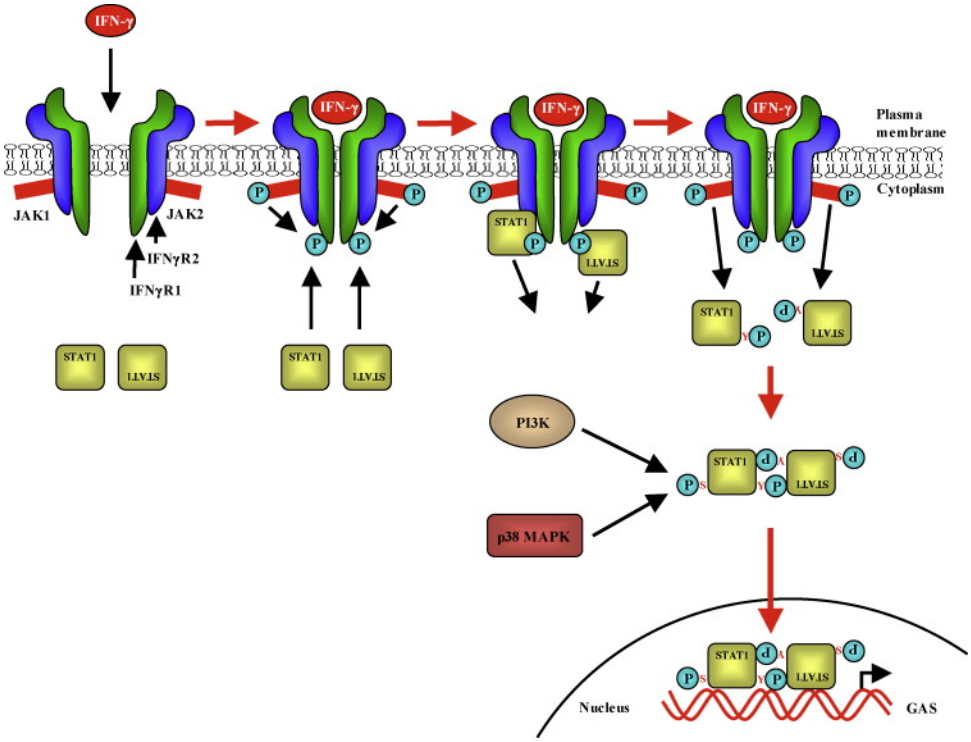


Figure 1.8: IFN-γ activates the JAK/STAT signalling pathway along with PI3Ks and MAPKs (McLaren and Ramji 2009).

The binding of IFN-γ causes the dimerisation of IFN-γR1 and IFN-γR2. The resulting receptor complex allows JAK-1 and -2 that are associated with the receptor to become phosphorylated and activated. STAT1 molecules become recruited and phosphorylated at tyrosine 701 by JAK-1 and -2. This promotes dissociation from the receptor complex, formation of a homodimer and translocation to the nucleus to perform transcriptional regulation. MAPKs and PI3Ks can also be activated by IFN-γR complex formation.

The role of IFN-γ in atherosclerosis is a widely debated topic because both pro- and anti-atherogenic properties have been observed in past studies that highlight a pleiotropic role for the cytokine (McLaren and Ramji 2009). In an early study, IFN-γ was shown to regulate foam cell formation during lesion initiation by inhibiting SR-A and CD36 expression in HMDMs and reducing the level of acetylated LDL uptake (Geng and Hansson 1992). However, most subsequent studies have shown stimulation of modified LDL uptake (McLaren and Ramji 2009). IFN-γ attenuated reverse cholesterol transport from THP-1 macrophages causing increased foam cell formation (Reiss et al. 2004). Also, work in THP-1 and HMDM cells showed an ERK dependent increase in modified LDL uptake and macrophage foam cell formation following stimulation with IFN-γ (Li et al. 2010). IFN-γ

also reduced the expression of key genes involved in the efflux of cellular cholesterol including apoE (Brand et al. 1993). The current *in vitro* evidence mainly supports a pro-atherogenic role for IFN-γ, although it does exhibit pleiotropic actions in some cases that have lead to slight controversy. One example is that the expression of LPL is reduced by IFN-γ stimulation; this action is athero-protective (Hughes et al. 2002). The evidence that has been presented during *in vivo* studies is clearer, it has been demonstrated that chronic administration of recombinant IFN-γ enhanced atherosclerosis in $apoE^{-/-}$ mice (Gupta et al. 1997; McLaren and Ramji 2009). Also, within $apoE^{-/-}$ and $LDLR^{-/-}$ mice, genetic ablation of IFN-γ, or the IFN-γ receptors reduced atherosclerosis (McLaren and Ramji 2009). A study demonstrated that blocking of IFN-γ function, using a soluble IFN-γR (mops up cytokine) expressing plasmid injected into $apoE^{-/-}$ mice prevented plaque progression, reduced lesion size and stabilised mature atherosclerotic plaques (Koga et al. 2007).

IFN-γ has also been shown to have an effect on ECs during the initiation and progression of atherosclerosis (McLaren and Ramji 2009). Expression of the monocyte adhesion molecule VCAM-1 was induced by IFN-γ along with ICAM-1 (Chung et al. 2002; Li et al. 1993). These findings suggest a pro-atherogenic role for the cytokine in relation to endothelial cells (Gupta et al. 1997). The cytokine also affects the biological activity of VSMCs (McLaren and Ramji 2009). Collagen synthesis by VSMCs is reduced by IFN-γ stimulation and there is less proliferation of VSMC from the media to the intima of the vessels (Gupta et al. 1997). Another effect on VSMC is that IFN-γ can prime the cells ready for apoptosis therefore promoting rupture of the plaque (Gupta et al. 1997).

It is clear from previous studies that the involvement of IFN-γ in atherosclerosis is very complex. However, it can be concluded that IFN-γ mainly acts in a pro-atherogenic way to promote the progression of the atherosclerotic lesion (McLaren and Ramji 2009).

1.3.2.3 - TL1A

The ligand to death receptor 3 (DR3) is a member of the TNF superfamily and is called TL1A (Migone et al. 2002). TL1A was originally thought to be predominantly expressed by endothelial cells, however more recent studies have suggested that TL1A is expressed by

variety of other cells including monocytes, macrophages, dendritic cells and T-cells (Migone et al. 2002; Pobezinskaya et al. 2011). DR3 is a death-domain containing tumour necrosis factor receptor (TNFR) superfamily member that is closely homologous to TNFR1. DR3 is preferentially expressed on lymphocytes and has been implicated in promoting many inflammatory diseases (Pobezinskaya et al. 2011). TNFR1 signaling has been very well characterised, activation of DR3 leads to formation of a signaling complex made up of tumour necrosis factor receptor type 1-associated death domain protein (TRADD), receptor interacting protein (RIP) and tumour necrosis factor receptor-associated factor 2 (TRAF2). These molecules are responsible for mediating the activation of MAPKs and NF-κB (Pobezinskaya et al. 2011).

Previously, in combination with IFN-γ, DR3 has been shown to have a role in atherosclerosis through stimulation of matrix degrading enzymes including MMP-9, potentially leading to de-stabilisation of the plaque (Kang et al. 2005). Another pro-atherogenic role for TL1A has been studied in relation to macrophage foam cell formation (McLaren et al. 2010a). The study demonstrated that TL1A promotes foam cell formation in human macrophages *in vitro* by increasing both acetylated and oxidized LDL uptake, by enhancing intracellular total and esterified cholesterol levels and reducing cholesterol efflux (McLaren et al. 2010a). This imbalance in cholesterol homeostasis was demonstrated to be orchestrated by TL1A-mediated changes in the mRNA and protein expression of several genes implicated in the uptake and efflux of cholesterol, such as SR-A and ABCA1 (McLaren et al. 2010a). Despite the evidence of TL1A acting to regulate plaque progression *in vitro*, the role of the TL1A-DR3 axis *in vivo* is yet to be determined.

1.3.2.4 - IL-17A

IL-17A is a potent proinflammatory cytokine produced by activated memory T-cells (Aggarwal and Gurney 2002). Upon studying the production of IL-17A by T-cells, a greater diversification within T-cells was discovered that previously was only covered by the T_h1/T_h2 paradigm. New studies that link the cytokines IL-23 and IL-17 to immune pathogenesis have led to the delineation of a new effector CD4 T-cell arm, T_h17 (Harrington et al. 2006). T_h17 represents a new T-cell lineage with important roles in the clearance of pathogenic bacteria and fungi (Harrington et al. 2006). The IL-17 family

comprises 6 members IL-17A, -B, -C, -D, -E and -F (Kolls and Linden 2004). The action of the cytokines is exerted through their receptor IL-17R, a single-pass transmembrane protein of approximately 130kDa. While the IL-17A cytokine is expressed only by T-cells, its receptor is expressed ubiquitously. The activation of the receptor by IL-17A generally results in the induction of other pro-inflammatory cytokines, through the activation of NF-κB (Moseley et al. 2003).

IL-17A has been regarded previously as pro-inflammatory, the cytokine has been shown to induce many mediators such as TNF-α and IL-1β (Gu et al. 2008). However, its role in atherosclerosis remains poorly understood and in some cases, controversial (Taleb et al. 2010). The few studies available on this topic have generated contrasting results, which could be attributed to different approaches used on various mouse models (Taleb et al. 2010). Several studies have suggested a pro-atherogenic role of IL-17A through the regulation of macrophage numbers, T_h1-related cytokines and chemokine expression (Butcher and Galkina 2011). However, two studies recently described anti-inflammatory effects of IL-17A on mouse plaque burden via possible regulation of aortic VCAM-1 expression and T-cell content (Butcher and Galkina 2011; Cheng et al. 2011; Taleb et al. 2009). To date studies are still producing contrasting conclusions (Butcher et al. 2012; Usui et al. 2012).

1.3.2.5 - IL-33

IL-33 is a recently characterised member of the IL-1 family of cytokines that promotes T_h2 type immune responses by signalling through the ST2L and IL-1RAcP dimeric receptor complex (Kakkar and Lee 2008). The biological actions of IL-33 were attenuated by introduction of naturally occurring soluble decoy variant of ST2 (sST2) within $apoE^{-/-}$ mice, the soluble receptor has affinity for IL-33 but no physiological action. These experiments highlighted the importance of ST2 action within IL-33 signal transduction during atherosclerosis (Miller et al. 2008). IL-33 can act both as a nuclear factor within cells and as a secreted traditional inflammatory cytokine (Kakkar and Lee 2008). The precise mechanism of how IL-33 is synthesised and trafficked through the cell has recently been mapped (Kakkar et al. 2012). As a cytokine, IL-33 is suggested to function as an alarmin that is released upon cellular necrosis or endothelial or epithelial cell damage (Kurowska-

Stolarska et al. 2011). As such, IL-33 targets multiple cell types thereby alerting the immune system to endogenous trauma such as physical stress or infection (Kurowska-Stolarska et al. 2011).

The activation of the ST2 receptor by IL-33 has also been shown to activate multiple signalling pathways including NF-κB, MAPK, and PI3K (Reviewed in Table 4.1). Recent studies indicate a protective role for IL-33 and ST2L in atherosclerosis (Miller and Liew 2011). Experimental studies have demonstrated that exogenous administration of IL-33 can induce a T_h1 to T_h2 shift within the plaques of an $apoE^{-/-}$ mouse model (Miller et al. 2008). Furthermore, $apoE^{-/-}$ mice treated for 6 weeks intraperitoneally with IL-33 exhibited significantly smaller atherosclerotic lesions than vehicle treated $apoE^{-/-}$ controls (Miller et al. 2008). IL-33 treated lesions showed reduced F4/80+ macrophage and CD3+ T-cell numbers, reduced T_h1 cytokine expression and increased T_h2 cytokine expression (Miller et al. 2008). Further evidence for an athero-protective role for IL-33 was provided by an *in vitro* study to ascertain the regulation IL-33 has on macrophage foam cell formation (McLaren et al. 2010b). In $apoE^{-/-}$ mice fed on a high fat diet, IL-33 treatment significantly reduced the accumulation of macrophage-derived foam cells in atherosclerotic plaques (McLaren et al. 2010b). IL-33 also reduced macrophage foam cell formation *in vitro* by decreasing acetylated and oxidized LDL uptake, reducing intracellular total and esterified cholesterol content and enhancing cholesterol efflux (McLaren et al. 2010b). These changes were associated with IL-33-mediated reduction in the expression of genes involved in modified LDL uptake, such as CD36, and simultaneous increase in genes involved in cholesterol efflux, including apoE (McLaren et al. 2010b). Furthermore, using bone marrow-derived macrophages from $ST2L^{-/-}$ mice, it was demonstrated that the IL-33 receptor, ST2L, is integral to the action of IL-33 on macrophage foam cell formation (McLaren et al. 2010b).

1.4 – Aims of Study

Atherosclerosis is recognised as a progressive, inflammatory disorder that leads to clinical endpoints such as heart attack, stroke and gangrene. CVD is one of the leading causes of death in western society and the prevalence is only predicted to increase. Plaque stability

is crucial in determining if a plaque causes the clinical endpoints that are observed, a plaque that has a high level of inflammation is usually at risk of rupture (Halvorsen et al. 2008). ADAMTS protease expression has recently been identified within lipid rich regions of the atherosclerotic plaque (Wagsater et al. 2008).

Some research has been carried out to ascertain the regulation of ADAMTS-4 and -5 expressions in human chondrocytes during OA and RA (Salter et al. 2010). However, the regulation of ADAMTS expression is poorly understood in relation to atherosclerosis**.** One study was carried out in 2008 that investigated the expression and regulation of ADAMTS proteases in macrophages, before and after differentiation, by IFN-γ, IL-1β and TNF-α (Wagsater et al. 2008). ADAMTS-4 and -8 were induced upon monocyte to macrophage differentiation and macrophage expression of ADAMTS-4, -7, -8 and -9 mRNA was enhanced upon stimulation with IFN-γ or TNF-α. On the other hand, IFN-γ attenuated the expression of ADAMTS-1 (Wagsater et al. 2008). Another study showed that TGF-β stimulation of human macrophages reduced ADAMTS-4 expression (Salter et al. 2011). These previous experiments highlight the importance of cytokine regulation over ADAMTS expression in atherosclerosis. The large amount of cytokines present within the plaque provides a wide scope for investigations into the regulation over ADAMTS expression from macrophages.

Also, the cellular consequences of ADAMTS protease activity are not fully characterised within the atherosclerotic plaque. The central hypothesised role of the ADAMTS proteases within the atherosclerotic plaque is cleavage of versican potentially leading to regulation of migration, proliferation, apoptosis and other cellular events within VSMC and macrophages (Salter et al. 2010). One study has shown that ADAMTS-7 regulated the migration of VSMC after balloon injury of rat arteries (Wang et al*.* 2009). The regulatory roles that ADAMTS proteases play during atherosclerotic disease progression are important to research.

The aims of this study were to increase the understanding of how ADAMTS proteases are regulated during atherosclerotic disease progression and also to further knowledge on

their cellular actions during disease progression. The specific questions are detailed below:

- Do cytokines regulate the expression of ADAMTS-1, -4 and -5 in human macrophages?
- Can cytokines act in synergy to regulate the expression of ADAMTS-1, -4 and -5 in human macrophages?
- What signal transduction mechanisms are responsible for the regulation of ADAMTS-1, -4 and -5 by IL-33 stimulation in human macrophages?
- Can the expression of ADAMTS-1, -4 and -5 be targeted by RNA interference to try and understand their cellular roles within atherosclerosis?

Chapter 2

Materials and Methods

2.1 - Materials

The materials that were used to carry out the project and where they were sourced from are listed in Table 2.1.

Table 2.1: The materials used in the project and the suppliers from which they were sourced

Material	Supplier
dNTP's; MMLV reverse transcriptase; RNasin ribonuclease inhibitor; Random hexamer primers; P-GEMT vector system; Luciferase gene reporter assay system.	Promega, UK
Ampicillin; Bromophenol blue; Ethidium bromide; Phorbol 12-myristate 13-acetate (PMA); Tween20; anti-β-actin antibody (A2228); SYBR green; X-ray film; DMSO; Accuspin tubes; DAPI mounting media; BacMAX 100 kit; RIPA buffer; Protease inhibitor cocktail.	Sigma, UK
Acrylamide.	Anachem, UK
Agarose; PCR reaction buffer.	Bioline, UK
10X TBE.	National Diagnostics, UK
RNeasy Plus Minikit; PCR purification kit; Miniprep kit; Maxiprep kit; Validated	Qiagen, UK

siRNAs; SuperFect; PolyFect.	
LB-media; LB-agar capsules.	GIBCO Laboratories, UK
THP-1; TREx-293; RAW264.7.	ECACC, UK
Tissue culture flasks; 6-well plates; 12-well plates; Cell scrapers; 10ml stripettes; 25ml stripettes; 50ml Falcon tubes.	Greiner Bio One, UK
TGF-β; IFN-γ; IL-33; IL-17A; TL1A; mIL-33; MCP-1.	Peprotech, UK
EDTA; Ethanol; Glycerol; Industrial methylated spirit; SDS; Tri-sodium citrate; Tris buffer; Filter papers.	Fisher Scientific, UK
Sterile 0.22μm filters; PVDF membrane.	Millipore, UK
ADAMTS-4 Antibody (PA1-1749).	Affinity Bioreagents, UK
PCR primers.	Sigma Genosys, UK
I block; CDP star reagent.	Applied biosystems, UK
NuPage™ Novex Gel Tank system; Blotting module; SDS-PAGE gels; MOPS running buffer; Transfer buffer; SOC-media; RPMI 1640 with GlutaMAX™ liquid; Penicillin and Streptomycin; Foetal calf serum; 2-mercaptoethanol; 1kb DNA molecular markers; DMEM high glucose media; 0.25% trypsin/EDTA; See-blue protein marker.	Invitrogen, UK
Donkey anti-goat alkaline phosphatase conjugate (AP); Chicken anti-goat AP; Goat anti-rabbit AP; Goat anti-mouse AP; Rabbit anti-goat AP; Anti-phospho-c-Jun Ser63 (sc-822); Anti-c-Jun (sc-1694); Anti-ADAMTS-1 (sc-25581); Anti-ADAMTS-5	Santa Cruz Biotechnology, USA

(sc-134952); Normal goat serum; Normal mouse IgG; Normal rabbit IgG; Normal rat IgG, Goat anti-rat FITC conjugate; Goat anti-rabbit TRITC conjugate.	
Anti-mouse CD36 antibody (FA-11).	Biolegend, UK
Buffy coats.	Welsh Blood Service, UK
Lymphoprep.	Axis Shield, Norway
X-Gal; IPTG solution.	Melford, UK
PCR tubes; PCR plates; PCR seals.	ELKay, UK
BCA protein assay.	Pierce, UK
Microplate Reader.	BioRad, UK
Opticon 2 PCR machine.	MJ Research, UK
PAP marking pen.	Abcam, UK
Clearene solution.	Leica, UK
Cell culture inserts; Companion plates.	Becton Dickinson, UK
Interferin™.	PolyPlus Transfection, USA
Anti-phospho p44/p42 Thr202/Tyr204 (9101); anti-total p44/p42 (9102); Anti-phospho p38 Thr180/Tyr182 (9211); anti-total p38 (9212); anti-phospho Akt Ser473 (9271); anti-total Akt (9272).	Cell Signaling, USA
Mouse anti-CD68 (EBM11); Mouse anti-Smooth muscle actin (1A4); Goat anti-rabbit-biotin (E0432); Goat anti-mouse-biotin (E0433); Negative control immunoglobulin.	Dako, UK
DiI-AcLDL; LDL.	Intracell, USA
Spin column RNA miniprep kit.	NBS Biologicals, UK

2.2 – Preparation of solutions and glassware

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

2.3 - Cell Culture Techniques

2.3.1 – Cell lines

2.3.1.1 - THP-1

THP-1 is a human monocytic leukaemia cell line. After differentiation with phorbol esters, THP-1 cells display many of the properties and characteristics of human monocyte-derived macrophages (Auwerx 1991; Kohro et al. 2004). Differentiated THP-1 cells are therefore a useful model, in which to study properties of human macrophages, including cell signalling and gene expression. THP-1 cells are a suspension cell line when left undifferentiated, although after addition of phorbol esters the cells become adherent to tissue culture flasks. However, the data that is obtained from studies that utilise THP-1 macrophages need to be interpreted with caution. The stimulation with PMA can upregulate a large number of genes and give false positive results or mask small regulatory actions (Park et al. 2007).

2.3.1.2 – RAW264.7

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

2.3.1.3 – T-REx293

T-REx293 is a human kidney cell line that shows stable expression of the tetracycline repressor protein. T-REx293 cell lines exhibit extremely low basal expression levels in the

repressed state and high expression upon induction with tetracycline. T-REx293 cells are an adherent cell line (Stanton et al. 2008).

2.3.2 - Maintenance of cell lines in culture

2.3.2.1 - THP-1 and RAW264.7

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

2.3.2.2 – T-REx293

T-REx293 cells were grown in Dulbecco's Modified Eagle medium (DMEM) with high glucose and GlutaMAX™. The medium was supplemented with 10% (v/v) HI-FCS and pen/strep. Both the HI-FCS and pen/strep were filter-sterilised by passing through a 0.2µm sterile filter before use. The cells were grown on large tissue culture flasks in a humidified incubator at 37°C, 5% (v/v) CO_2.

2.3.3 - Sub-culturing of cells

2.3.3.1 – THP-1

THP-1 cells were 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 1:30 ratio so that growth back up to 60% confluency occurred in approximately one week. For experiments, cells between passage two and ten were used.

2.3.3.2 – RAW264.7

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 scrapping. 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.

2.3.3.3 – T-REx293

T-REx293 cells were sub-cultured when they reached approximately 80% confluency on the surface of the culture flask. The cells were removed from the flask by adding 0.25% (v/v) trypsin/ethylenediaminetetraacetic acid (EDTA) solution to cover the cell monolayer. Cells were incubated at 37°C, 5% (v/v) CO_2 until they became visibly detached from the flask. The resulting solution was transferred into a Falcon tube and centrifuged at 100g for 5min. The media and trypsin solution was aspirated from the pellet and the cells were re-suspended in fresh pre-warmed media containing 10% (v/v) HI-FCS and pen/strep. Cells were placed in new tissue culture flasks at a ratio of 1:12 and grown up at 37°C in a humidified, 5% (v/v) CO_2 incubator.

2.3.4 - Preserving and storing cells

THP-1, RAW264.7 and T-REx293 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.

2.3.5 - Thawing frozen cells

Cells from liquid nitrogen were defrosted by placing in a water bath at 37°C. Cells were transferred to a polypropylene tube containing 10ml HI-FCS and centrifuged at 100g for

5min. After re-suspension in fresh pre-warmed medium containing 10% (v/v) HI-FCS, cells were plated out into tissue culture flasks and cultured as normal.

2.3.6 - Counting cells

To count the THP-1, RAW264.7 and T-REx293 cells, a haemocytometer was used. After centrifugation at 100g for 5min, cells were re-suspended in 3-5ml of culture medium containing 10% (v/v) HI-FCS and pen/strep. The haemocytometer was covered with a precision ground cover-slip and 7µl of cell suspension was applied. The numbers of cells in a 5 x 5 grid were counted and the number of cells/ml was calculated by multiplying the number of cells in the grid by 10^4.

2.3.7 – Seeding cells for cytokine stimulation

2.3.7.1 – THP-1

For a standard RNA based experiment, $5x10^5$ cells were placed in each well of a 12-well plate and supplemented with 1ml of culture medium with 10% (v/v) HI-FCS and pen/strep. During experiments cells were incubated at 37°C in a humidified, 5% (v/v) CO_2 incubator. For a PMA time course experiment, 0.16µM PMA was added for the following time points: 24, 48, 72 and 96hrs. For cytokine stimulation, the cells were differentiated using 1.6µM PMA for 24 hours; the cytokines were added for a further 24hrs before harvesting the RNA. The cytokines that were used are shown in Table 2.2, along with their concentration. For western blot experiments, $1x10^6$ cells were added to each well of a 6 well plate and supplemented with 1ml of culture medium containing 10% (v/v) HI-FCS; the addition of PMA and cytokines was the same as the RNA based experiments.

Table 2.2: Cytokines used and their concentration

Cytokine	Concentration used	Reference for concentration
TGF-β	30ng/ml	(Salter et al. 2011)
IL-33	10ng/ml	(McLaren et al. 2010b)
TL1A	100ng/ml	(McLaren et al. 2010a)
IL-17A	100ng/ml	(Shahrara et al. 2009)
IFN-γ	1000U/ml	(Li et al. 2010)
mIL-33	10ng/ml	Used the same concentration as human IL-33

2.3.7.2 – RAW264.7

For a standard RAW264.7 experiment, $5x10^5$ cells were placed in each well of a 12-well plate and supplemented with 1ml of culture medium with 10% (v/v) HI-FCS and pen/strep. During experiments cells were incubated at 37°C in a humidified, 5% (v/v) CO_2 incubator. The cells were left to adhere to the surface of the 12-well plate overnight. The cytokines used to stimulate the RAW264.7 cells for 24hrs were mouse cytokines in all cases (Table 2.2).

2.3.8 - Human monocyte-derived macrophage (HMDM) primary cell culture techniques

When preparing HMDM cultures, a buffy coat was obtained from the Welsh Blood Service. The monocytes were purified and plated out on 12-well plates. Firstly, lymphoprep solution was warmed to room temperature and 15ml was added to accuspin centrifuge tubes. The tube was centrifuged at 1000g for 1min to place the lymphoprep below the filter. Then, 30ml of blood from the buffy coat was poured onto the filter and the tube was centrifuged at 1000g for 30min at room temperature. The mononuclear cells were then collected and transferred to a new centrifuge tube with an equal volume of ice-cold PBS-0.4% (w/v) tri-sodium citrate, this was centrifuged at 1000g for 5min at 4°C. The pelleted cells were then resuspended in 10 ml of 0.2% (v/v) saline solution and incubated on ice for 30secs. Following this, 10ml of 1.6% (v/v) saline was added with an immediate centrifugation at 1000g for 5min at 4°C. This red blood cell lysis step was repeated to remove more red blood cells. The resultant pellet was then collected and

washed 6-8 times with 10ml of ice-cold PBS-0.4% (w/v) tri-sodium citrate to remove contaminating platelets. The cells were then plated out in RPMI media supplemented with 10% (v/v) HI-FCS and pen/strep. The cells were left to differentiate for 10 days before use in experiments; the media was changed every two days during the differentiation.

2.4 - RNA/DNA related techniques

2.4.1 - Isolation of total RNA

Total RNA was isolated from cells using a RNeasy Plus Mini Kit (Qiagen) or a Spin Column RNA Miniprep Kit (NBS Biologicals). Before using the kit, the medium was aspirated from the cells, and they were washed with 1ml of phosphate buffered saline (PBS). If the cells were in suspension then they were centrifuged at 100g for 5min to form a cell pellet before this PBS wash step. Cells were then re-suspended in 350µl RLT buffer (from kit) and then supplemented with 10µl/ml β-mercaptoethanol. At this stage the lysate could be stored at -80°C for up to 6 months, or used immediately for RNA extraction. The resulting lysate was homogenised by passing it through a QIAshredder (Qiagen). The lysate was then transferred to a genomic DNA elimination column (in kit). The purified lysate was transferred to a silica-based membrane spin column to bind the RNA. Contaminants were washed away using RW1 wash buffer (in kit) before the RNA was eluted.

The RNA concentration and quality of RNA was determined using a NanoDrop 2000 spectrophotometer according to the manufacturer's instructions. The integrity of the RNA was analysed by size-fractioning a small amount on a 1.5% (w/v) agarose gel (section 2.4.4).

2.4.2 – Reverse Transcription

RNA (1µg or 0.5µg if the concentration of RNA isolated was low) was mixed with random hexamer primers (200pmol) and sterile water to a total volume of 13.5µl. This was incubated at 72°C for 5min and immediately cooled on ice. The following reagents were added to the reaction:

1µl of deoxyribonucleotide (dNTP) mixture containing 10mM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP);
4µl of 5X molony murine leukaemia virus (MMLV) reverse transcriptase buffer;
0.5µl of recombinant RNase inhibitor;
1µl of MMLV reverse transcriptase.

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

2.4.3 - Polymerase chain reaction (PCR)

Once the cDNA had been manufactured it was possible to carry out a PCR reaction, the primer sequences that were used are shown in Table 2.3. The composition of the PCR reactions used in the RT-PCR reactions is shown in Table 2.4, along with the reaction conditions in Table 2.5.

Table 2.3: PCR primers for analysis of gene expression

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Reference
RPL13A	CCTGGAGGAGAA GAGGAAAGAG	TTGAGGACCTCT GTGTATTTGT	126	(Vandesompele et al. 2002)
GAPDH	GAAGGTGAAGGT CGGAGTC	GAAGATGGTGAT GGGATTTC	226	(Yamanishi et al. 2002)
ADAMTS-1	GCACTGCAAGGC GTAGGAC	AAGCATGGTTTC CACATAGCG	90	(Cross et al. 2005)
ADAMTS-4	TCACTGACTTCC TGGACAATGG	GGAAAGTCACAG GCAGATGCA	81	(Cross et al. 2005)
ADAMTS-5	CACTGTGGCTCA CGAAATCG	CGCTTATCTTCT GTGGAACCAAA	93	(Cross et al. 2005)
mADAMTS-1	GCTCATCTGCCA AGCCAAAG	CTACAACCTTGG GCTGCAAAA	110	(Wen et al. 2006)
mADAMTS-4	ATGGCCTCAATC CATCCCAG	AAGCAGGGTTGG AATCTTTGC	107	Primer Bank
Apo-E	TTCCTGGCAGGA TGCCAGGC	GGTCAGTTGTTC CTCCAGTTC	270	(Wang et al. 1989)
LPL	GAGATTTCTCTG TATGGCACC	CTGCAAATGAGA CACTTTCTC	276	(Wang et al. 1989)
ERK-1	GCAGGACCTGAT GGAGACTGAC	CCAGAATGCAGC CCACAGAC	344	QPPD
ERK-2	GCGCTACACCAA CCTCTCGT	CACGGTGCAGAA CGTTAGCTG	377	QPPD
p38 MAPK	GTGGTACAGGGC TCCTGAGA	TATGCATCCCAC TGACCAAA	79	-
c-Jun	TCCAAGTGCCGA AAAAGGAAG	CGAGTTCTGAGC TTTCAAGGT	78	Primer Bank
JNK-1	TCTGGTATGATC CTTCTGAAGCA	TCCTCCAAGTCC ATAACTTCCTT	127	Primer Bank

JNK-2	GAAACTAAGCCG TCCTTTTCAGA	TCCAGCTCCATG TGAATAACCT	207	Primer Bank
Smad2	CCCATCAAATTC AGAGAGGTTC	TCACTTAGGCAC TCAGCAAAAA	144	-
PI3K-δ	ACCTGAACTTCC CTGTGTCC	CACTGAGCATGT GGAAGAGC	99	Primer Bank
PI3K-γ	TCTGATGGATAT TCCCGAAAGCC	CTCACCCACTGG AAGTTTTTGAT	111	Primer Bank
mβ-actin	GTGCCACCAGAC AGCACTGTGTTG	TGGAGAAGAGCC TATGAGCTGCCTG	202	(McLaren et al. 2010c)

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

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

Reagent	Amount
Forward Primer	0.5 μl
Reverse Primer	0.5 μl
dNTPs	1 μl
Buffer	5 μl
"In house" Taq polymerase	0.25 μl
Sterile Water	32.75 μl
cDNA	10 μl

Table 2.5: Amplification conditions for RT-PCR

PCR Step	ADAMTS-1	ADAMTS-4	ADAMTS-5
Initial Melting	95°C for 5 min	95°C for 5 min	95°C for 5 min
Annealing	60°C for 60 sec	64°C for 60 sec	62°C for 60 sec
Extension	72°C for 60 sec	72°C for 60 sec	72°C for 60 sec
Melting	95°C for 30 sec	95°C for 30 sec	95°C for 30 sec
Final Extension	72°C for 10 min	72°C for 10 min	72°C for 10 min
Number of Cycles	30	30	30

2.4.4 - Agarose Gel Electrophoresis

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

Table 2.6: Composition of reagents used in gel electrophoresis

Reagent	Composition
10 x TBE	0.89M Tris borate, 890mM boric acid and 20mM EDTA, ddH_2O, pH 8.3
5 x DNA loading dye	1 x TBE, 50% (v/v) glycerol and 2.25% (w/v) bromophenol blue

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

2.4.5 - PCR purification

The RT-PCR products were purified before performing any ligations. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). The Kit was used according to the manufacturer's instructions.

2.4.6 - Ligation

The ligation was carried out using a pGEM-T vector system and a 2X rapid ligation buffer (Promega). The ligation was carried out according to the manufacturer's instructions.

2.4.7 - Preparation of competent cells

The protocol used in the preparation of competent cells was a modified version of calcium dependant DNA uptake by bacteria. This method was first proposed by Mandel and Higa (Mandel and Higa 1970). Firstly, 5ml of preheated (37°C) LB medium was inoculated with a single bacterial colony of *E.coli* strain DH5α and incubated at 37°C for 12-18hr while shaking (300rpm). Then, 9.9ml of LB medium (pre-warmed to 37˚C) was inoculated with 0.1ml of this culture and incubated for a further 2hr under the same conditions. Cells were pelleted by centrifugation at 3000g for 5min at 4°C, re-suspended in 5ml of 50mM $CaCl_2$ and kept on ice for 25min. Cells were then pelleted as described above and re-suspended in 1ml 50mM $CaCl_2$. Competent cells were either kept on ice before transformation or mixed with 40% (v/v) glycerol, aliquoted and stored at -80°C.

2.4.8 - Transformation of competent cells

Competent cells were thawed on ice prior to transformation. Plasmid DNA was added to 50µl of competent cells and kept on ice for 30min. The cells were then heat shocked at 42°C for 20secs and immediately placed on ice for a further 2min. Then, 950µl of pre-warmed SOC-media (Invitrogen) was added and the cells were incubated at 37°C with shaking (200rpm) for 1hr. To select ampicillin resistant bacteria colonies, the cells were spread on LB-agar plates containing 100µg/ml ampicillin. In addition, 20µl of (10mg/ml) X-Gal and (8mg/ml) IPTG solution (Melford) was also spread onto the plates to carry out blue/white screening. Plates were incubated at 37°C overnight and analysed for ampicillin resistance and blue/white colonies.

These colonies were then selected and transferred into 5ml cultures containing ampicillin and incubated overnight at 37°C while shaking (200rpm). The cultures contained 5ml of LB media and ampicillin (100µg/ml).

2.4.9 - Plasmid extraction and purification

2.4.9.1 – Small-scale preparation of plasmid DNA (Mini-prep)

LB-medium (5ml) containing 100µg/ml ampicillin was inoculated with a single colony of transformed *E.coli* and incubated overnight at 37°C with shaking (200rpm). Plasmid extraction was carried out using a MiniPrep Kit (QIAGEN). The procedure was carried out following the manufacturer's instructions. The concentration and the purity of the resulting eluted DNA samples were checked on the NanoDrop2000 by assessing ratios of 260:280nm absorbances (Thermo Scientific).

2.4.9.2 – Large-scale preparation of plasmid DNA (Maxi-prep)

LB-medium (10ml) containing 100µg/ml ampicillin was inoculated with a single colony of transformed *E.coli* and incubated for 8hr at 37°C with shaking (200rpm). This was then used to inoculate 500ml fresh LB medium with ampicillin (100µg/ml) and this was incubated at 37°C with shaking (200rpm) overnight. Plasmid extraction was carried out using a QIAPrep Spin Maxiprep Kit (Qiagen). The procedure was carried out following the manufacturer's instructions. The concentration and the purity of the resulting eluted DNA samples were checked on the NanoDrop2000 by assessing ratios of 260:280nm absorbances (Thermo Scientific).

2.4.10 – DNA sequencing

M13 forward and reverse primers that were able to amplify the pGEM®-T vector were used for sequencing to detect correctly transformed colonies. The Molecular Biology Support Unit, of Cardiff University, performed the sequencing.

2.4.11 – Real time quantitative PCR (Q-PCR)

One problem with traditional end-point RT-PCR reactions is that the endpoints could lie out of the exponential phase of the PCR reaction. This means that in some cases the semi-quantification of the starting template could be inaccurate (Ginzinger 2002). Q-PCR is a technique that allows reliable detection and measurement of products generated

during each cycle of the PCR reaction. The amount of PCR product produced is directly proportional to the amount of template prior to the start of the PCR process. To accomplish this level of real time measurement it is necessary to detect the accumulation of PCR products after each cycle of the reaction. An instrument is required that can perform the thermo-cycling, while being adapted to record the results during each PCR cycle in real time (Ginzinger 2002). In order for the technique to work a fluorescent dye needs to be added to the reactions that emits signal when it is bound to dsDNA. SYBR green is a dye that intercalates with dsDNA, similar to ethidium bromide (Giulietti et al. 2001). The dye has undetectable fluorescence in its free form, but fluoresces on binding to dsDNA. SYBR green is not specific to primer sets so care must be taken that the reaction conditions are highly optimised, therefore reducing contaminations such as primer dimers (Giulietti et al. 2001). Q-PCR reactions were set up using SYBR Green JumpStart™ Taq Readymix™ for quantitative PCR (Sigma Aldrich). The reactions were then analysed using an Opticon 2 PCR machine (MJ Research). The composition of the reactions is detailed in Table 2.7.

Table 2.7: The composition of a 25µl Q-PCR reaction

Reagent	Amount (µl)
SYBR Green Ready mix	12.5
Forward Primer	0.5
Reverse Primer	0.5
cDNA	1
Water	10.5
Total	25

The reagents were mixed and plated into a 96 well plate and the program was set according to the optimised PCR conditions (Table 2.5).

The comparative Ct (ΔΔCt) method was used for the analysis of the Q-PCR data (Livak and Schmittgen 2001). The ΔΔCt method relies on measuring the number of reaction cycles it takes for the fluorescent signal from the reaction to pass a pre-set threshold level (Ct

value) within the exponential phase of the amplification. The Ct value of the gene of interest is then compared relative to the Ct value of a control housekeeping gene to normalise the data (Livak and Schmittgen 2001). The ΔΔCt method relies on two assumptions, firstly that the expression of the housekeeping gene does not vary under experimental conditions and secondly, that the PCR efficiencies for each primer set used in the analysis are similar (Ginzinger 2002). The housekeeping genes that were used were GAPDH and RPL13A. GAPDH is an enzyme that has a critical involvement in glycolysis. The expression is commonly shown to be stable during many experimental conditions and therefore GAPDH has been widely used as a housekeeping gene for Q-PCR analysis (Legastelois et al. 1998; Wu et al. 1997). RPL13A is a ribosomal protein that has previously been shown to be reliable standard in a number of expression studies by Q-PCR (Foldager et al. 2009; Pombo-Suarez et al. 2008; Salter et al. 2011).

After the Q-PCR reaction had been performed and the threshold level set, the Ct values were then collected and analysed on Microsoft Excel.

2.5 - DNA Transfections

2.5.1 - Superfect™ Transfection

DNA transfection work was carried out in RAW264.7 cell lines using conditions that have been previously used in our laboratory on other cell lines such as U937 and Hep3B. The cells were grown in RPMI containing 10% (v/v) HI-FCS and pen/strep. Confluent RAW264.7 cells were scraped from the culture dish and seeded as previously described (section 2.3.7.2). Prior to transfection the medium was replaced with 0.5ml of fresh medium supplemented with 10% (v/v) HI-FCS and pen/strep.

Transient transfection was carried out using SuperFect™ according to the manufacturer's instructions (Qiagen) with minor modifications. Briefly, the transfection mix was prepared in 50µl medium that contained no HI-FCS and no pen/strep. SuperFect™ reagent and the plasmid DNA of interest (containing a promoter region of the gene of interest, linked to

the firefly-luciferase reporter gene) were added in a 3:1 ratio. Plasmid maps and further information on each plasmid can be found in the Appendix. SuperFect™ has a dendrimer structure with positively charged amino groups that interact with the phosphate backbone of DNA to form a complex that can be taken up into cells by endocytosis. The total transfection mix was allowed to incubate at room temperature for 10min before being added to 0.6ml of medium containing 10% (v/v) HI-FCS and pen/strep. The complete mix was added dropwise to the cells. Cytokine treatment was carried out 1hr following transfection (37°C in a humidified, 5% (v/v) CO2 incubator). Cells were then incubated overnight before being harvested using 1X Passive Lysis Buffer (Promega).

2.5.2 – Preparation of cell extracts for determination of reporter gene activity

To prepare cell extracts for analysis, the medium was aspirated from the RAW264.7 cells and a PBS wash step was carefully performed. After the PBS had been aspirated from the cells, 200µl of 1X Passive Lysis Buffer was added directly to the cell monolayer and left to incubate for 15min at room temperature. Cells were then scrapped into the lysis buffer and transferred to a micro-centrifuge tube and centrifuged at top speed for 5min. The supernatant was either stored at -20°C or used immediately for the measurement of luciferase activity.

2.5.3 – Luciferase assay

Luciferase activity of the samples was determined using a Dual-Luciferase® Reporter Assay System (Promega). Firefly luciferase is an enzyme that catalyses the activation of luciferin by ATP to produce an anhydride intermediate which then reacts with oxygen to produce oxyluciferin, CO_2 and light (Smale 2010). Luciferase substrate (0.1ml) and sample (0.1ml) were added to a black, flat optically clear bottomed 96-well plate (Corning). Luciferase activity was determined using a LUMIstar OPTIMA (BMG Labtech). Measurements were taken in duplicate and luciferase counts were normalised to the protein concentration (µg/µl) of each sample (section 2.7.3).

2.6 – siRNA Transfections

2.6.1 – siRNA transfection with Interferin™

siRNA transfections were carried out using validated siRNAs against target mRNAs (Table 2.8). Stock solutions were prepared from lyophilised siRNAs according to the manufacturer's instructions (Qiagen and Invitrogen). siRNA transfection was carried out in THP-1 cells prior to differentiation with 0.16µM PMA. Confluent THP-1 cells were harvested by centrifugation at 100g for 5min. The pellet was re-suspended in the appropriate volume of medium supplemented with 10% (v/v) HI-FCS and no antibiotics. Cells (500,000) were seeded into 12-well plates and incubated at 37°C in a humidified, 5% (v/v) CO_2 incubator for 4hr prior to transfection. siRNA transfection was carried out according to the manufacturer's instructions (Polyplus Transfection) with minor modifications. Briefly, a transfection mix was prepared in 0.1ml of antibiotic-free and HI-FCS-free RPMI-1640 medium. The mix contained 7.5nM siRNA and 9µl of Interferin; it was briefly micro-centrifuged and left to incubate at room temperature for 20min. The complete mixture (100µl) was then added dropwise to the cells and then they were placed at 37°C in a humidified, 5% (v/v) CO_2 incubator for 24hr. Following transfection (24hr), cells were differentiated using 0.16µM PMA for 24hr. Cells were then subjected to cytokine stimulation for the requisite time period before being harvested for RNA extraction (section 2.4.1) or protein extraction (section 2.7.1 – 2.7.3).

Table 2.8: siRNAs used during the course of this study

Target mRNA transcript	NCBI accession number(s) of target	SI reference number / Catalogue number
Negative control	N/A	AM4611
GAPDH	NM_002046	AM4624
c-Jun	NM_002228	SI00300580
ERK-2	NM_002745	SI00300755
JNK-1	NM_002750	SI02758637
JNK-2	NM_001135044	SI02222920
Smad-2	NM_001003652	SI02757496

2.7 – Protein Analysis

2.7.1 – Preparation of protein extracts using Gel Sample Buffer

When performing experiments for protein analysis $1x10^6$ THP-1 cells were seeded per sample in 6-well tissue culture plates. Following experimentation, the medium was aspirated from the well and the cells were washed with 1ml of ice cold PBS. The PBS was then aspirated from the well and the cells were lysed with 100µl of Gel Sample Buffer (Table 2.9) by scraping. The lysate was transferred to a 1.5ml micro-centrifuge tube and centrifuged at top speed for 5min. Lysates were stored at -80°C or used immediately for SDS-PAGE followed by western blotting (section 2.7.4 – 2.7.7).

Table 2.9: Composition of stock solutions used for protein analysis by SDS-PAGE and western blotting

Solution	Composition
Gel Sample Buffer	63mM Tris HCl pH 6.8, 10% (v/v) glycerol, 5% (v/v) β-mecaptoethanol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol Blue
NuPAGE® 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
NuPAGE® Transfer Buffer	50mM Bis-tris propane, 50mM Bicine, 20% (v/v) methanol
I-BT solution	500ml PBS-Tween, 1g Tropix I-Block, 500µl of 5% (v/v) sodium azide
PBS-Tween	10L ddH_20, 100 PBS tablets, 20ml Tween20
Coomassie Blue	45% (v/v) methanol, 10% (v/v) glacial acetic acid, 3g/L coomassie brilliant blue, ddH_2O
Ponceau S	0.1% (w/v) Ponceau S, 5%(v/v) acetic acid, ddH_2O

2.7.3 – Determination of protein concentrations

Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce). Samples were processed in 96-well plates and a BSA standard curve was prepared for each assay using the appropriate buffer. Samples were routinely diluted 1 in 50 and placed into the 96-well plate in duplicate (150µl). Reagents A, B and C, provided in the kit, were mixed according to the manufacturer's instructions and added to each well (150µl). The plate was covered and left to incubate at 37°C for 2.5hr. A Model 680 Micro-plate Reader (Biorad) was used to measure the absorbance of each sample at a wavelength of 570nm.

2.7.4 – SDS polyacrylamide gel electrophoresis

Protein samples were prepared as previously described (section 2.7.1 and 2.7.2). Prior to loading the protein samples onto the gel, the samples where first boiled at 100°C for 5min. SDS-PAGE was carried out using a Novex NuPAGE Gel Electrophoresis System (Invitrogen). Within this system the samples were analysed on NuPAGE 4–12% Bis-Tris Gels (1.0-mm thick, 10-well). These are pre-cast polyacrylamide gels designed to give optimal separation of small- to medium-sized proteins under denaturing conditions. Unlike traditional tris-glycine gels, NuPAGE Bis-Tris gels have a neutral pH environment that minimizes protein modifications. The tank was filled with NuPAGE MOPS SDS Running Buffer (Invitrogen) and the gels were placed within the tank. An approximately equal amount of protein sample was added to each well (30µl), along with a protein standard, the gel was subjected to electrophoresis for 1hr at 200V, 400mA. Efficiency of SDS-PAGE separation was checked at this stage by staining with Coomassie Blue.

2.7.5 – Western blotting

After SDS-PAGE, the gel was removed from its casing and arranged into an XCell II™ Blot Module (Invitrogen) with Immobulin-P PVDF membrane (Millipore) that had been previously activated in methanol. The blot module was prepared wet in NuPAGE® Transfer buffer, and the module was then topped up with this buffer. The transfer was carried out at 30V, 300mA for 80min. Transfer efficiency was checked at this stage by staining the PVDF membrane with Ponceau S solution.

2.7.6 – Immuno-detection of proteins

Following blotting, the membrane was removed from the sandwich and washed once with PBS-Tween for 5min. The wash was removed and the blot was then placed in I-BT solution (Table 2.9) for 1hr while shaking; this step was carried out to block the membrane. After removal of the I-BT solution the primary antibody was added (optimised dilution in I-BT, Table 2.10) and left overnight at 4°C while shaking. Following

the primary antibody incubation the membrane was washed three times for 5min with PBS-Tween. After the wash steps were complete, the secondary antibody was added for 1hr at room temperature (diluted in I-BT, Table 2.10). After 1hr the membrane was again washed three times for 5min with PBS-Tween.

Table 2.10: Antibodies and dilutions used for western blotting

Protein	Primary Antibody Species	1° Dilution	Secondary Antibody	2° Dilution	Protein Size
β-actin	Mouse	1:10,000	Goat Anti Mouse (AP)	1:5000	42KDa
ADAMTS-4	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	53 and 68 KDa
ApoE	Goat	1:1000	Rabbit Anti Goat (AP)	1:5000	34KDa
Phospho c-Jun	Mouse	1:1000	Goat Anti Mouse (AP)	1:5000	39KDa
Total c-Jun	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	39KDa
Phospho p44/p42	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	42 and 44 KDa
Total p44/p42	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	42 and 44 KDa
Phospho p38	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	43 KDa
Total p38	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	43 KDa
Phospho Akt	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	60 KDa
Total Akt	Rabbit	1:1000	Goat Anti Rabbit (AP)	1:5000	60 KDa

2.7.7 – Detection of chemiluminescence

After the PVDF membrane had been probed with the relevant antibodies, Tropix CDP-Star® 0.25mM (Applied Biosystems) was added to the blot surface (0.5ml). Chemiluminescent detection was carried out according to the manufacturer's protocol with minor modifications. Briefly, the chemiluminescent detection reagent is cleaved by alkaline phosphatase that is linked to the secondary antibody. This results in a luminescent signal that is proportional to the amount of protein present. Membranes were placed in contact with the x-ray film in a light proof cassette (Genetic Research Instrumentation) for varying exposure times. The film was developed using a developing machine (Agfa).

2.7.8 – Semi-quantitative densitometric analysis of western blots

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

2.8 – Immunohistochemistry

2.8.1 – Human Plaques

2.8.1.1 – Slide preparation

The sections were obtained from the Bristol Coronary Biobank. They were obtained from cadaver hearts donated to research. Coronary arteries had been removed from the heart tissue and cut into 5mm sections. Sections were formalin fixed, paraffin embedded and then cut into 5µm sections to be placed on slides for staining.

2.8.1.2 – De-waxing and re-hydration

Slides were firstly placed in three washes of Clearene solution (Leica); each wash was performed for 5min. Following this step to de-wax the sections, a re-hydration step was carried out. The slides were placed in a series of graduated ethanol washes for 5min each. The concentrations used were 100%, 90% and 70% (v/v) ethanol.

2.8.1.3 – Blocking endogenous peroxidase activity

Sections were washed twice in ddH_2O for 5min and then 0.3% (v/v) H_2O_2 was added for 7min. This step inhibits endogenous peroxidase activity that could interfere with the DAB staining at a later stage in the protocol.

2.8.1.4 – Target retrieval

Sections were again washed twice in ddH_2O for 5min and the slides were placed into a reservoir containing citrate buffer (10mM Sodium Citrate, 0.05% (v/v) Tween20, pH 6.0). The reservoir was put into a microwave on full power for 6min. The level of citrate buffer was checked and adjusted at this stage if needed. After topping up, the reservoir was again put into the microwave for 6min. The reservoir was then allowed to cool for 30min at room temperature.

2.8.1.5 – Antibodies

Sections were rinsed in PBS three times after removal from the citrate buffer, then allowed to air dry. Sections were encircled using a PAP pen (Abcam); this enabled multiple samples and antibodies to be used on a single slide. Also the amount of antibody that had to be used was reduced. Before incubation with the primary antibody the sections were blocked with 10 % (v/v) serum (same species as secondary) for 30min at room temperature. The remaining block solution was removed and 50µl of the primary antibody solution (Table 2.11) was applied to the sections. The sections were then incubated in a humidified chamber with the lid on at 4°C overnight. Slides were taken and placed into coplin-jars, and then three washes of PBS were carried out to remove any unbound primary antibody. Sections were then allowed to air dry prior to addition of the biotinylated secondary antibody solution (Table 2.11). The secondary antibody solution was left to incubate in a humidified chamber for 30min at room temperature.

2.8.1.6 – DAB and haematoxylin staining

Any unbound secondary antibody was washed off by three PBS washes performed in coplin-jars. 50µl of extravidin-horseradish peroxidase (1:400 of stock in PBS 1% (w/v) BSA) was added to the slides for 30min at room temperature, in order to attach the enzyme horseradish peroxidase onto the secondary antibody. The resulting slides were again washed three times in PBS (coplin-jars). Following this wash step, the DAB solution was added and left for a sufficient time for stain development. The sections were then counter stained using haematoxylin and de-hydrated by putting into 70%, 90% and 100% (v/v) ethanol for 5min each. Then the slides were placed in clearene solution (section 2.8.1.2) and allowed to air dry.

2.8.1.7 – Microscope examination and Imaging

Slides were mounted with a coverslip and DPX mountant (Leica). The slides were then examined using a Leica Light Microscope.

Table 2.11: Antibodies used during immunohistochemistry on human plaques

Target	Antibody Species	Dilution	Secondary Antibody (1:400)
CD68	Mouse	1:50	Goat anti-mouse (Biotynylated)
Smooth Muscle Actin	Mouse	1:50	Goat anti-mouse (Biotinylated)
ADAMTS-1	Rabbit	1:50	Goat anti-rabbit (Biotinylated)
ADAMTS-4	Rabbit	1:50	Goat anti-rabbit (Biotinylated)
ADAMTS-5	Rabbit	1:50	Goat anti-rabbit (Biotinylated)

2.9 – Macrophage Migration Assays

2.9.1 – THP-1 cells

2.9.1.1 – Seeding cells and setting up the trans-well system

Macrophage migration assays were carried out using BD Falcon ™ Cell Culture Inserts (8µm). The inserts were placed inside BD Falcon™ Companion Tissue Culture Plates. THP-1 cells were counted and seeded (section 2.3.6 and 2.3.7) into the top of the cell culture insert in 0.5ml of medium supplemented with 10% (v/v) HI-FCS and pen/strep. They were then infected with adenoviral vectors (section 2.10.2.1) and differentiated for 48hrs using 0.16µM PMA. There was 1ml of medium in the bottom well of the trans-well system during this differentiation period. A chemokine (MCP-1 (20ng/ml)) was added into the bottom chamber for 24hr and then insert was then ready for processing.

2.9.1.2 – Processing cell culture insert and mounting onto slide

Cells that hadn't undergone migration were removed from the top of the trans-well; this was achieved by gentle scrapping using a cotton bud. The insert could then be rinsed in PBS. The cells that had undergone migration (on the reverse side of the insert) where then fixed in 90% (v/v) ethanol for 5min at room temperature. The inserts could be stored in 70% (v/v) ethanol at 4°C for later use or used immediately at this stage.

2.9.1.3 – Microscope examination and image analysis

The membranes where removed from the casing of the cell culture insert by cutting with a sharp scalpel. The resulting membrane was then placed onto a microscope slide and mounted with a cover-slip and anti-fade DAPI mounting medium (Sigma). The slides were examined and imaged on a fluorescent microscope (Olympus BX61). Image analysis was performed using CellProfiler Cell Image Analysis Software.

2.10 – Designing an adenoviral vector to deliver specific shRNAs

2.10.1 – Designing and making the adenovirus

2.10.1.1 – Cloning shRNAs into pAdZ-5 (mIR155)

The oligonucleotides were designed using an algorithm on the Invitrogen website (RNAi Designer). The sequences of oligonucleotides used for each target is presented in Chapter 6.

2.10.1.2 – Creating competent SW102s

Firstly, SW102s were inoculated with pAdZ5-mIR155 in 5ml of LB medium containing ampicillin (50µg/ml) and chloramphenicol (25mg/ml). The 5ml cultures were incubated overnight at 32°C. Following this, 0.5ml of the overnight culture was inoculated into 25ml of LB media supplemented with ampicillin (50µg/ml). The resulting suspension was incubated in a shaking incubator to an OD_{600} of 0.6. Once the culture had reached an OD_{600} of 0.6 it was heated at 42°C for 15min to induce the lambda red proteins, followed by cooling on ice for 15-20min. The culture was then centrifuged at 1000g for 5min at 0°C. The supernatant was poured off and the pellet was re-suspended in 1ml of ice cold ddH_2O followed by further adding 25ml of ddH_2O. The washing step was then repeated (care being taken with the pellet). The SW102s were then aliquoted with the oligonucleotide DNA and pre-cooled for 5min on ice. The samples were then electroporated at 2.5kV in 0.2cm cuvettes. After electroporation the bacteria were recovered in 5ml LB media for 4hr in a shaking incubator at 32°C. From these cultures, 50µl was plated onto LB agar plates (10g/L tryptone, 5g/L yeast extract, 5% (w/v) sucrose, 15g/L agar, 12.5µg/ml chloramphenicol, 80µg/ml X-gal, 2mM IPTG) and were left to grow colonies for 30-48hr at 32°C. Correct colonies were identified on plates and selected to be grown up for the following stages.

2.10.1.3 – Minipreps

Plasmid extraction and purification was carried out using a Miniprep kit (Qiagen) with a slightly modified version of the manufacturer's protocol. To prepare the cultures for the

minipreps positive colonies were used to innoculate 5mls of LB media. The 5ml cultures contained chloramphenicol (25µg/ml) and they were left to grow for 16-20hr at 32°C. The cells were then centrifuged at 100g for 5min, the supernatant was removed and the pellet re-suspended in 250µl buffer P1 (in kit). To this, 250µl of buffer P2 (in kit) was added and the sample was incubated at room temperature for 5min. 250µl of buffer N3 (in kit) was added and the sample was mixed well and then centrifuged at 1000g for 10min. The supernatant was transferred to a new tube and a DNA precipitation step was carried out using isopropanol (750µl). The resulting sample was centrifuged at 1000g for 10min (4°C) to produce a visible DNA pellet. The supernatant was discarded and the pellet was washed with 70% (v/v) ethanol. The pellet was air-dried and re-dissolved in buffer EB from the Qiagen kit.

2.10.1.4 – Restriction Digest

DNA obtained from the mini-prep (section 2.10.1.3) was subjected to a restriction digest to check if the recombination of the plasmid had occurred correctly. For this, 8µl of DNA was mixed with 1µl of reaction buffer (NEB) and 1µl of BamH1 (NEB) and was incubated for 1hr at 37°C. The resulting DNA samples were then size-fractionated on an agarose gel to visualize the banding pattern (section 2.4.4). The AdZ-5 vector gives bands at 18, 11, 7.7, 2.5, 1.7, 0.8, and 0.6Kbp. The 2.5 and 1.7Kbp bands are from the SacB/LacZ/Amp cassette, so in positive colonies these should have disappeared.

2.10.1.5 – Sequencing

DNA sequencing was carried out by The Molecular Biology Support Unit, School of Biosciences, Cardiff University. The following primers were used in this process (Table 2.13).

Table 2.13: Primers used in DNA sequencing of AdZ-5 vector

Target Region of Vector	Sequence of Primer (5'-3')
CMV Promoter	AATGTCGTAACAACTCCG
CMV PolyA	ACCTGATGGTGATAAGAAG

2.10.1.6 – Maxi-prep

Once the colony had been confirmed to be correct by restriction digest and DNA sequencing then a culture was grown up to purify a larger amount of DNA (30µg). Firstly, a single colony was inoculated into a 5ml LB culture containing chloramphenicol (25µg/ml) and incubated for 6-8hr at 32°C in a shaking incubator. The resulting culture was then diluted into 250ml LB and chloramphenicol (25µg/ml) and incubated overnight at 32°C (shaking). The DNA was prepared using the BacMAX 100 kit (Sigma). At the end of the manufacturer's protocol the DNA was re-suspended in 100µl 10mM Tris-HCl pH8.5. The concentration of the DNA was between 200-400ng/µl.

2.10.1.7 – Generating RAds

Firstly, $2x10^6$ TREx-293 cells were seeded into a small tissue culture flask the day before transfection. Then, 4µg of recombinant vector DNA was prepared in 100µl DMEM with no HI-FCS or antibiotics. PolyFect (Qiagen) 40µl; was added and the mixture was incubated at room temperature for 10min. While the DNA complexes were incubating, the media on the TREx-293 cells was aspirated and changed (3ml DMEM and 10% (v/v) HI-FCS containing pen/strep). Then, 1ml of DMEM and 10% (v/v) HI-FCS containing pen/strep was added to the DNA complexes and then the total mixture was added to the cells. The transfections were incubated at 37°C, 5% (v/v) CO_2 for 24hr. After 24hr the media was changed and the transfections were returned to the incubator. Viral plaques formed after 7-10 days. To make a useable amount of virus more cells had to be infected, so $6x10^6$ TREx-293 were seeded into a large tissue culture flask. Infected cells from the initial transfection were then added and the flasks were cultured for 3-10 days until cells had formed plaques. The virus was then extracted from the cells using a PBS and tetrachloroethylene extraction and stored at -80°C in 100µl aliquots. Briefly, cells were re-suspended in 1ml of PBS and 1ml of tetrachloroethylene was added to lyse the cells. The resulting interface was shaken vigorously and centrifuged at 1000g for 20mins. The top clear layer containing the virus was then removed and aliquoted.

2.10.1.8 – Titering of adenoviruses

To work out the titre of the adenovirus produced, $5x10^5$ cells were first seeded into each well of a 12-well plate and allowed to adhere overnight (37°C, 5% (v/v) CO_2). The next

day, serial dilutions were prepared of the viral stock. The 10^{-4} and 10^{-5} dilutions were added to the TREx-293 cells and these were incubated for 48hr (37°C, 5% (v/v) CO_2). After this incubation the media was aspirated and the wells were allowed to air-dry. Then, 1ml of ice cold 50%/50% (v/v) acetone/methanol was added and allowed to incubate for 10min at -20°C. The acetone/methanol was aspirated and the cells were washed 3 times with PBS containing 1% (w/v) BSA. A goat anti-adenovirus primary antibody (Chemicon) was added in a 1:5000 dilution (PBS containing 1% (w/v) BSA) and was rocked for 1hr at 37°C. The primary antibody was then aspirated and the cells were washed 3 times in PBS containing 1% (w/v) BSA. Then, two secondary antibodies were added, donkey anti-goat and chicken anti-goat (Abcam). They were added in a 1:1000 dilution in PBS containing 1% (w/v) BSA. The secondary antibodies were then removed and the cells were again washed 3 times with PBS containing 1% (w/v) BSA and then DAB staining was performed for 10min. The cells were then counted on a light microscope (Leica) as the number of infected cells per field of view at a magnification of X10. The numbers of plaque forming units/ml (pfu/ml) were calculated using:

$$\frac{(Infected\ cells\ per\ field\ \boldsymbol{X}\ 150)}{(Virus\ Volume\ (ml)\ \boldsymbol{X}\ dilution\ factor)}$$

2.10.2 – Using the adenovirus to infect human cells during experiments

2.10.2.1 – THP-1 cells

THP-1 cells were counted and seeded as previously described (section 2.3.3.1, 2.3.6 and 2.3.7). The adenovirus was added to the cells at a multiplicity of infection (MOI) of 100 (previously optimised in our laboratory). The infection was left for 2.5hr at 37°C, 5% (v/v) CO_2, and then differentiation was performed with 0.16µM PMA. The cells were then incubated for 48hr at 37°C, 5% (v/v) CO2, followed by cytokine stimulation.

2.10.2.2 – HMDMs

HMDMs were prepared and seeded as previously described (section 2.3.8). After the 10 day differentiation period the adenovirus was added to the cells with the same MOI as the THP-1 cells (section 2.10.2.1). Here an assumption was made that the number of

primary cells in each well was similar to that of the counted THP-1 cells. The infection was left for 48hr at 37°C, 5% (v/v) CO_2, and then the normal cytokine stimulation was performed.

2.10.2.3 – Testing adenoviral (shRNA) knockdown efficiency

Adenovirus shRNA knockdown efficiency was tested by Q-PCR (section 2.4.11) and western blot analysis (section 2.7).

2.11 – Macrophage cholesterol uptake assays

For a standard cholesterol uptake assay, $5x10^5$ cells were 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. During experiments cells were incubated at 37°C in a humidified, 5% (v/v) CO_2 incubator. The cells were differentiated using 0.16µM PMA for 24hrs prior to starting the assay. The cells were then further incubated for 24hrs with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyane perchlorate (DiI)-labeled acetylated LDL (DiI-AcLDL) in RPMI 1640 at 37°C along with the addition of the required cytokines (Table 2.2). DiI-AcLDL uptake was analyzed by flow cytometry on a FACSCanto (BD Biosciences, Oxford, U.K.) flow cytometer with at least 10,000 events acquired for each sample. DiI-AcLDL uptake is represented as a percentage of the untreated control, which was arbitrarily assigned as 100%.

2.12 - Statistical analysis of data

Data sets were tested for normality using the Shapiro-Wilk test. Single comparisons of data were analysed using a student's t-test (two-tailed, paired) or Wilkcoxon t-test. For multiple comparisons one-way ANOVA was mainly used along 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. Results were regarded as significant if ≤P=0.05.

Chapter 3

ADAMTS - Immunohistochemistry and Cytokine Regulation

3.1. Introduction

3.1.1 – Atherosclerosis and Plaque Stability

Rupture of the atherosclerotic plaque leading to thrombosis is one of the most common causes of mortality in western societies (McLaren et al. 2011). Clinical complications of atherosclerosis are only observed when the mature atherosclerotic plaque undergoes rupture (Lusis 2000). Therefore, regulation of plaque stability could potentially reduce the incidence of the clinical consequences attributed to atherosclerosis (Libby and Aikawa 2002). Many inflammatory mediators regulate cellular events, such as collagen synthesis, matrix degradation and apoptosis, that may enhance the plaques vulnerability to rupture (Halvorsen et al. 2008). Migration and proliferation of VSMCs is very important in the formation and maintenance of the fibrous cap and the process is highly regulated by various factors (Newby 2006). Macrophages, endothelial cells and T-cells play a role in regulating VSMC migration by secreting chemokines and growth factors (Newby et al. 2009). The stability of the plaque is maintained by having a balance between ECM synthesis and breakdown, if the balance is lost then the stability of the plaque is lowered. A plaque in this chronic pro-inflammatory unstable condition is at high risk of rupture (Halvorsen et al. 2008). It has been suggested that several proteases play a vital role in the regulation of plaque stability by modulating VSMC migration, proliferation and viability (Newby 2006). Some of these proteases are also able to break down the fibrous cap ECM, therefore weakening the plaque (Newby 2006). The expression of proteases from macrophages within the plaque is very tightly regulated by a range of growth factors and cytokines (Newby 2008). One major group of enzymes that has been extensively studied are the MMPs (Newby et al. 2009). A dual role for MMPs in atherosclerotic

plaque development has been suggested (Newby 2005); the action of the MMPs has been extensively reviewed in the General Introduction. 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. The action of the proteases depends on their identity and regulation (Newby 2005). Specific MMP knockout and inhibition studies have been employed to try and understand how specific MMPs act during atherosclerosis, these studies are sometimes inconsistent (Newby et al. 2009). Further research into the action of specific MMPs needs to be carried out. However, an additional area to explore would be to investigate the action of other proteases acting to remodel the atherosclerotic plaque ECM.

3.1.2 – ADAMTS Proteases

3.1.2.1 - Introduction

ADAMTS proteases are a family of 19 proteins that share a similar domain pattern and substrate range; they are structurally related to the ADAM and MMP protease families and have been extensively reviewed in the General Introduction and elsewhere (Porter et al. 2005; Salter et al. 2010; Tortorella et al. 2009). The founding member of the ADAMTS proteases was first cloned, identified and named in a study carried out in 1997 (Kuno et al. 1997), from here the family has grown to 19 members (Porter et al. 2005; Salter et al. 2010). ADAMTS proteases are non-membrane bound proteins that act on a wide variety of ECM substrates including pro-collagen, proteoglycans, hyalectans and cartilage oligometric matrix protein (Jones and Riley 2005). The physiological roles of the ADAMTS proteases are now beginning to be understood; a role in cartilage turnover and also in atherosclerosis has been suggested (Salter et al. 2010; Tortorella et al. 2009).

3.1.2.2 – ADAMTS and Atherosclerosis

MMPs have been suggested to be major regulators of the atherosclerotic process through re-modeling of the plaque ECM (Newby et al. 2009). As the protein families are structurally related, the role for ADAMTS proteins in atherosclerosis could be similar to that of the MMPs (Salter et al. 2010). ADAMTS-1, -4, -5 and -8 have been shown to be expressed within human atherosclerotic plaques, and macrophages were identified as the

major contributors towards ADAMTS expression in the disease (Jonsson-Rylander et al. 2005; Wagsater et al. 2008). ADAMTS proteases were also expressed in VSMC and endothelial cells, but to a lower extent than macrophages and foam cells (Jonsson-Rylander et al. 2005; Wagsater et al. 2008). ADAMTS-1 expression has been studied in various wild type mouse tissues and was shown to be at the highest level in the aorta (Jonsson-Rylander et al. 2005). ADAMTS-4 mRNA was present in $LDLR^{-/-}ApoB^{100/100}$ aortas before any atherosclerotic lesions were visible and the level of expression increased as the lesions became more advanced (Wagsater et al. 2008). These findings suggest that ADAMTS proteases are present within human and mouse atherosclerotic plaques and therefore could have a regulatory role during the disease process.

The central hypothesised role of the ADAMTS proteases within the atherosclerotic plaque is the cleavage of versican potentially leading to the regulation of migration, proliferation, apoptosis and other cellular events within VSMC and macrophages (Salter et al. 2010). Versican is a large ECM proteoglycan that is highly expressed in atherosclerotic plaques (Wight 2002). The highly interactive nature of the molecule gives a basis for its importance in the disease progression (Wight 2002). The role of versican cleavage within atherosclerosis is a complicated one; expression has been detected within all three arterial wall layers and is interestingly up-regulated during all forms of vascular disease (Lemire et al. 2007). Versican is likely to provide structure and strength to the vasculature through its interaction with hyaluronan (Wight 2002). Versican-hyaluronan complexes are necessary for the migration and proliferation of VSMCs following wounding and also serve as an attachment for macrophages and lymphocytes, suggesting that the complexes may influence the retention of inflammatory cells (Evanko et al. 1999). The chondroitin sulphate chains of versican can interact with chemokines and adhesion molecules that could influence recruitment and migration of vascular cells including macrophages (Hirose et al. 2001; Kawashima et al. 2000). The chondroitin sulphate chains also interact with LDL particles in advanced lesions leading to increased LDL uptake in both VSMCs and macrophages (Llorente-Cortés et al. 2002). These findings suggest that cleavage of versican and other ECM proteoglycans by ADAMTS proteases during atherosclerosis could have regulatory control over important cellular aspects of the disease.

The ADAMTS protease family is sub-divided into four classes based on structural and functional similarities, the classes are named the proteoglycanases, the procollagen-n-peptidases, von Willebrand cleaving factor and those ADAMTS proteases whose function remains to be fully elucidated (Salter et al. 2010). The proteoglycanases are the sub-class that is most relevant to atherosclerosis. The proteoglycanases ADAMTS-1, -4, -5, -8, -9 and -15 possess anti-angiogenic activity, and out of these ADAMTS-1, -4, -5 and -9 are able to cleave aggrecan (Cross et al. 2005). Most importantly in relation to their activity during atherosclerosis ADAMTS-1, -4, -5 and -9 are also able to cleave versican (Cal et al. 2002; Cross et al. 2005; Sandy et al. 2001; Westling et al. 2004).

3.1.3 – Cytokine Regulation of ADAMTS Proteases

Atherosclerosis is a chronic inflammatory disease of the vascular wall. A plethora of cytokines act in many different ways to regulate the initiation, progression and rupture of the atherosclerotic plaque (Libby 2002; McLaren et al. 2011; Tedgui and Mallat 2006). Previous studies have provided evidence that the expression of specific ADAMTS proteases can be regulated by cytokine stimulation (reviewed in the General Introduction). These findings suggest that ADAMTS protease expression could be potentially regulated by cytokines that are present within the atherosclerotic plaque. Due to the number of cytokines that are produced within the atherosclerotic plaque, studies of their actions on ADAMTS expression are a potentially important area of research.

3.2 – Experimental Aims

There were three primary aims of this first experimental chapter. Firstly, it was of interest to study the expression of ADAMTS-1, -4 and -5 in human atherosclerotic plaque sections. ADAMTS-1, -4 and -5 were specifically chosen as it has been demonstrated that ADAMTS-1 is expressed within mouse atherosclerotic plaques and ADAMTS-4 and -5 are expressed in human carotid lesions (Jonsson-Rylander et al. 2005; Wagsater et al. 2008). It was of interest to create a detailed expression profile of ADAMTS-1, -4 and -5 within human atherosclerotic plaques.

The second experimental aim of this chapter was to design an *in vitro* human macrophage model that could be used to study the regulation of ADAMTS-1, -4 and -5 expressions. An experimental model utilising the THP-1 cell line was developed and characterised. THP-1 monocytes differentiate into macrophages upon stimulation with PMA and are widely used when studying various aspects of atherosclerosis due to their conservation of responses compared to primary cultures and *in vivo* (more detail in Materials and Methods). The expression of ADAMTS-1, -4 and -5 was analysed during THP-1 differentiation in order to find the optimal length of differentiation for future experiments.

The third experimental aim was to utilise the THP-1 macrophage cellular model to study how some selected cytokines affected the expression of ADAMTS-1, -4 and -5. The cytokines that were chosen along with the important studies linking their activities to atherosclerosis are described in Table 3.1 (further explained in General Introduction). The experimental strategies that were used during this chapter to accomplish these three major aims are outlined in Figures 3.1 - 3.3.

Table 3.1: Cytokines chosen for investigation

Cytokine	Links with atherosclerosis	Reference
TGF-β	Decreases foam cell formation. Decreases ADAMTS-4 expression.	(Salter et al. 2011; Singh and Ramji 2006a)
IFN-γ	Many pro-inflammatory roles, slightly pleiotropic. Reduces ADAMTS-1 expression. Increases ADAMTS-4, -7, -8 and -9 expressions.	(Jonsson-Rylander et al. 2005; McLaren and Ramji 2009; Wagsater et al. 2008)
TL1A	Increases MMP-9 expression and stimulates foam cell formation.	(Kang et al. 2005; McLaren et al. 2010a)
IL-17A	Potential role in atherosclerosis suggested by many mouse studies, some contradictory results. Mainly pro-atherogenic.	(Butcher and Galkina 2011; Taleb et al. 2010)
IL-33	Reduction of plaque burden in apoE$^{-/-}$ mouse model system. Reduction of foam cell formation. Converts T_h1 to T_h2 responses.	(Kakkar and Lee 2008; McLaren et al. 2010b; Miller and Liew 2011; Miller et al. 2008)

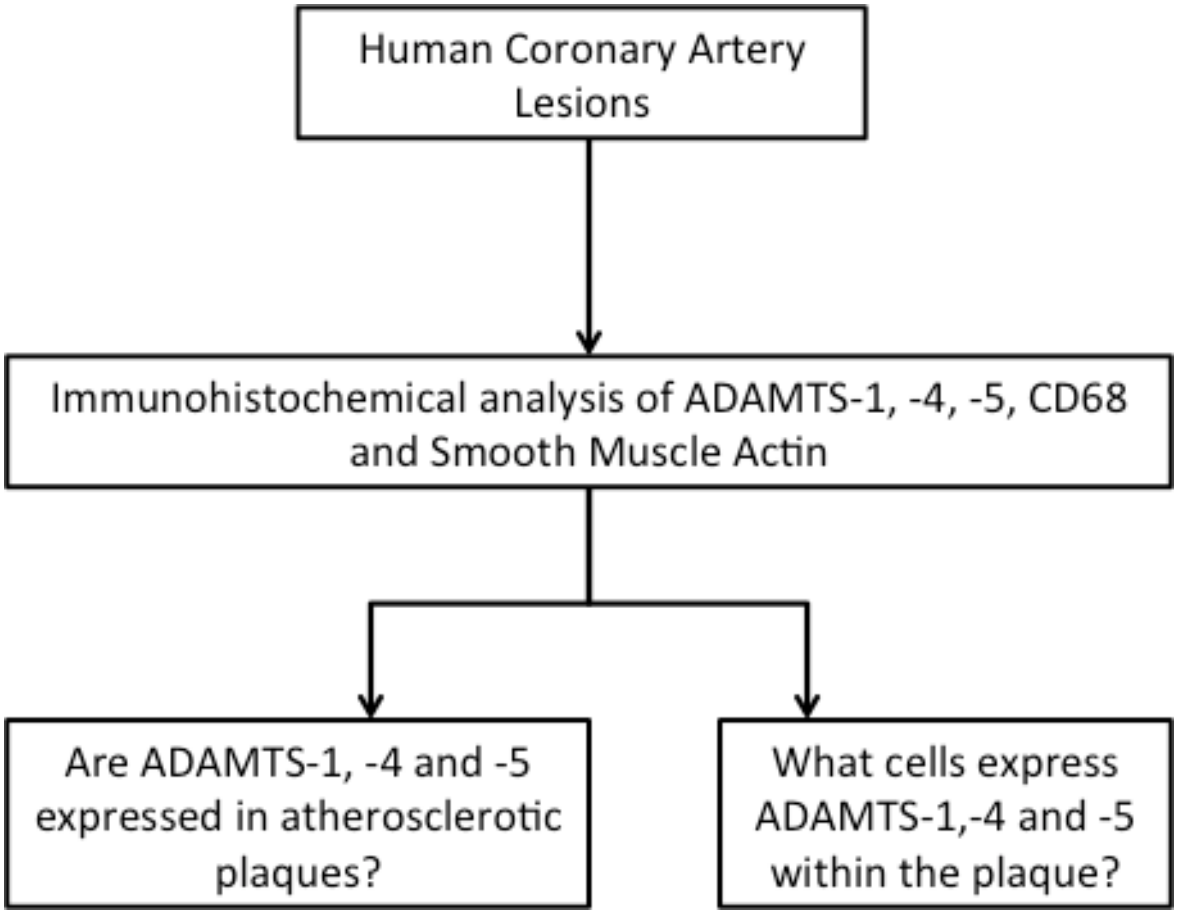


Figure 3.1: Immunohistochemistry Experimental Strategy

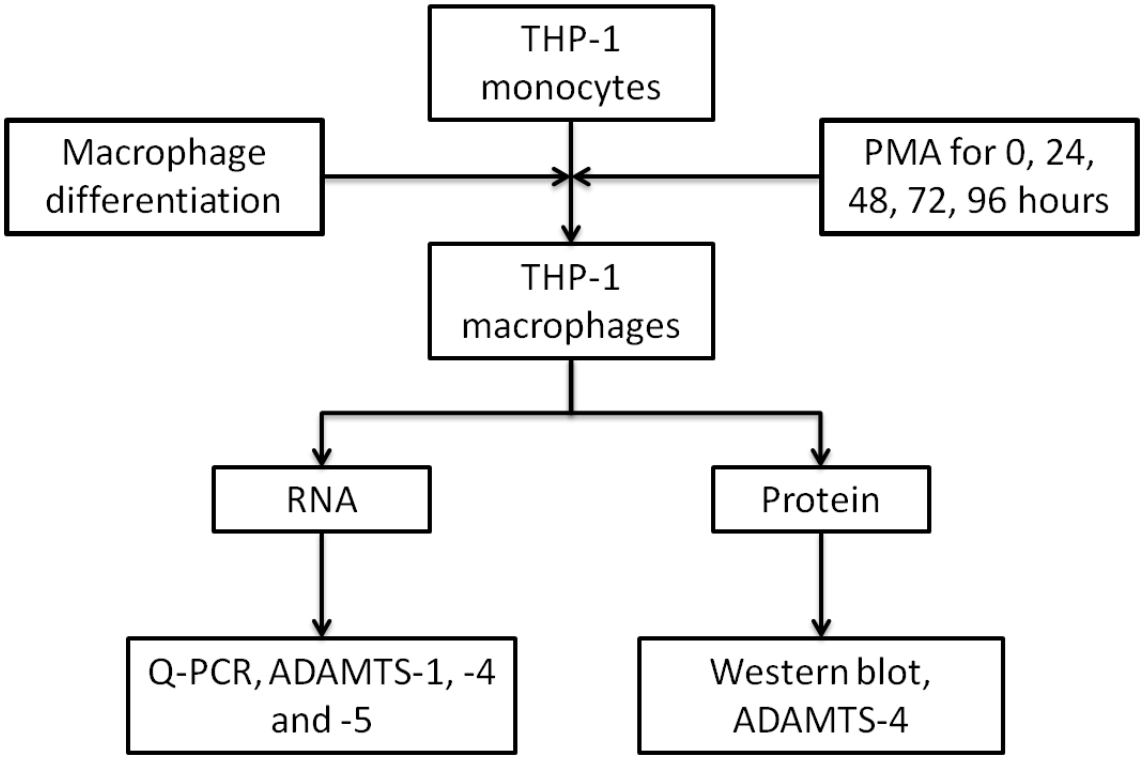


Figure 3.2: Strategy for developing an *in vitro* model using THP-1 cells

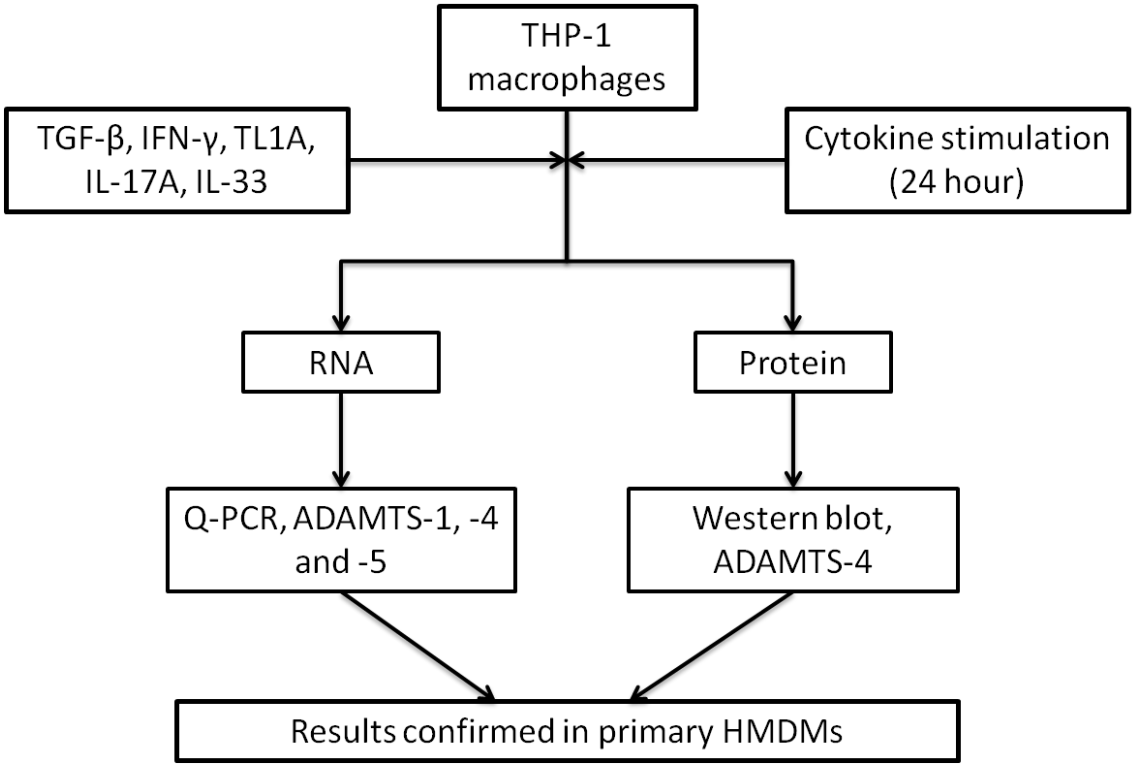


Figure 3.3: THP-1 Cytokine Stimulation Experimental Strategy

3.3 – Results

3.3.1 – Immunohistochemistry

3.3.1.1 – Human Plaques

All human immunohistochemical analysis was carried out at the Bristol Heart Institute and tissues were obtained from the Bristol Coronary Biobank. The staining for ADAMTS-1, -4 and -5 was carried out on serial sections alongside CD68 and smooth muscle actin. CD68 is a well-studied macrophage marker and smooth muscle actin is a well-studied VSMC marker (Lee et al. 2011). This 'dual staining' approach allowed areas of co-expression to be identified. The inclusion of cell specific markers allowed identification of which cell types were involved in expressing ADAMTS-1, -4 and -5 within the atherosclerotic plaque. The results from the immunohistochemistry are presented in Figure 3.4.

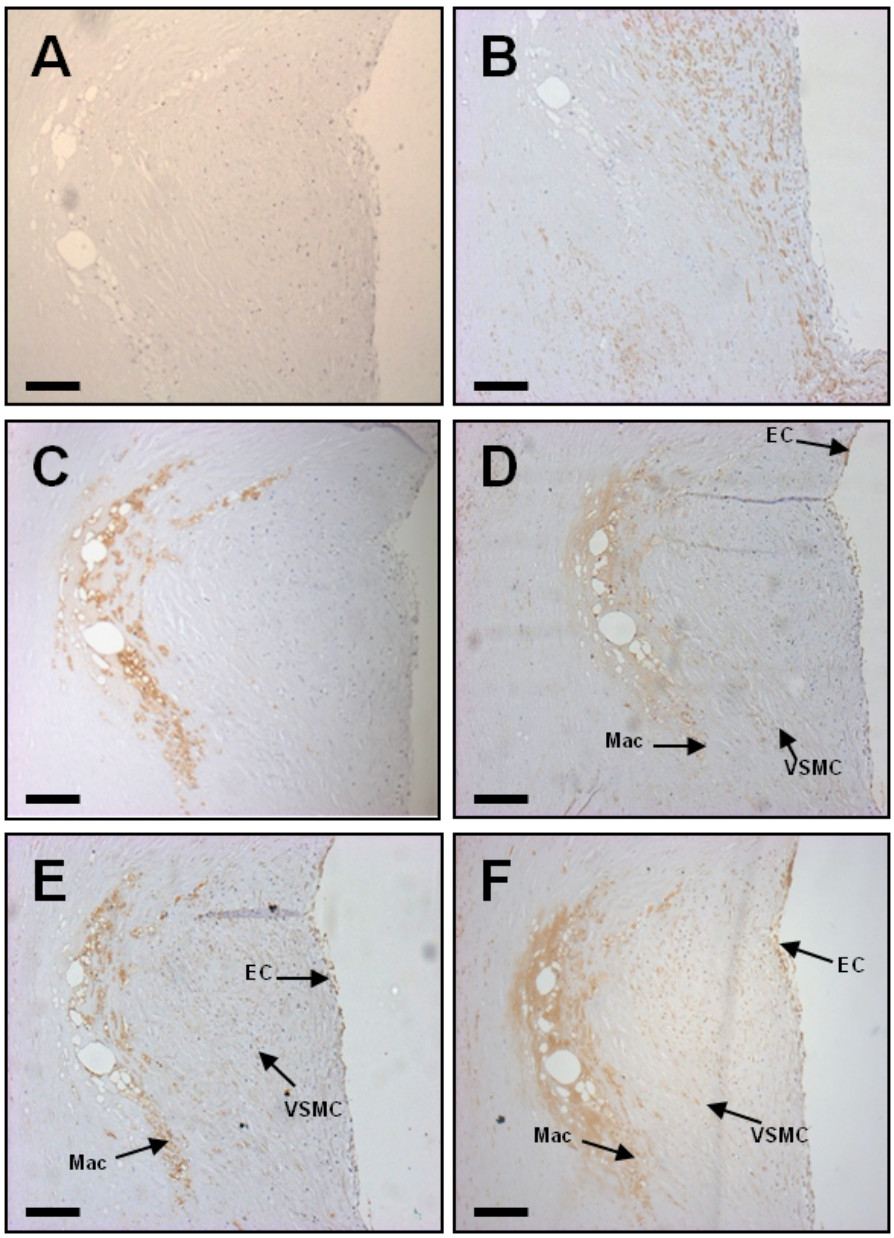


Figure 3.4: ADAMTS isoforms were expressed in human atherosclerotic lesions

Serial sections taken from human coronary arteries were stained using immunohistochemistry and visualized by DAB staining. Sections were stained for (A) Negative control IgG, (B) Smooth Muscle Actin, (C) CD68, (D) ADAMTS-1, (E) ADAMTS-4 and (F) ADAMTS-5. The sections were visualized using the X10 objective on a light microscope. Arrows point to locations of macrophages (Mac), Vascular Smooth Muscle Cells (VSMC) and Endothelial Cells (EC). Scale bar = 50µm. Images are representative of 6 independent sections.

When sections were stained using a negative isotype control there was no background staining produced by non-specific interactions with the tissue (Figure 3.4, panel A). When the plaque section was stained against smooth muscle actin the DAB staining was concentrated in the fibrous cap region of the plaque and in the media layer of the coronary artery (Figure 3.4, panel B). The CD68 staining indicated that macrophages and macrophage foam cells were concentrated in the shoulder regions of the plaque and also in and around the lipid rich core (Figure 3.4, panel C). ADAMTS-1, -4 and -5 were all expressed within the human atherosclerotic lesion (Figure 3.4, panels D, E and F). When looking at the distribution of their expression it was observed that they were mainly expressed by macrophages. They were also expressed by VSMC and endothelial cells but to a much lesser extent (Figure 3.4, arrows marked VSMC and EC).

3.3.2 - Optimisation of PCR conditions

The second and third experimental objectives of this chapter were to study the expression of ADAMTS-1, -4 and -5 in macrophages before, and after stimulation with cytokines that have important roles during atherosclerosis (Table 3.1). Before carrying out any PCR-based assays the initial integrity of macrophage RNA preparations was analysed by resolving an aliquot on a 1.5% (w/v) agarose gel. As expected for good quality RNA, the relative intensity of the 28S rRNA band was approximately twice that of the 18S rRNA band (Figure 3.5). RNA of this quality was produced throughout this thesis and hence these data are not shown from herein.

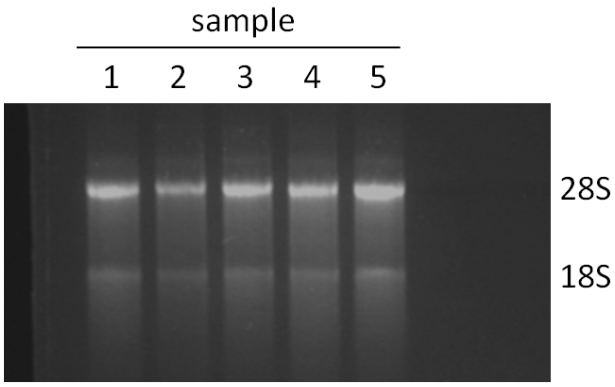


Figure 3.5: **Determination of RNA integrity**

Five separate total RNA preparations from differentiated THP-1 cells were subjected to gel electrophoresis to check RNA integrity.

3.3.2.1 – ADAMTS Primer conditions

Primer sequences for ADAMTS-1, -4 and -5 were sourced from previous publications (Materials and Methods) or from websites called PrimerBank or QPPD and ordered from Genosys (Sigma Aldrich). The primer sets were chosen to amplify a specific product of around 100bp in size. This is the optimal size of product when carrying out a Q-PCR assay (Ginzinger 2002). The reaction conditions and amounts of each reagent required were not listed within the publications, so these were optimised in order to obtain maximum and specific output from the PCR. The amount of reagents to use in each reaction was well established in our laboratory, but the annealing temperatures differ with each primer set that is used. For each set of primers a range of annealing temperatures were tested using conventional end point RT-PCR to find the optimum. It was found that when using the primers for ADAMTS-1 the optimal annealing temperature was 60°C, ADAMTS-4 was 64°C and ADAMTS-5 was 62°C. The products of the optimised PCR reactions are shown in Figure 3.6. Negative control PCR reactions with no cDNA template were included to check for contamination of samples with genomic DNA.

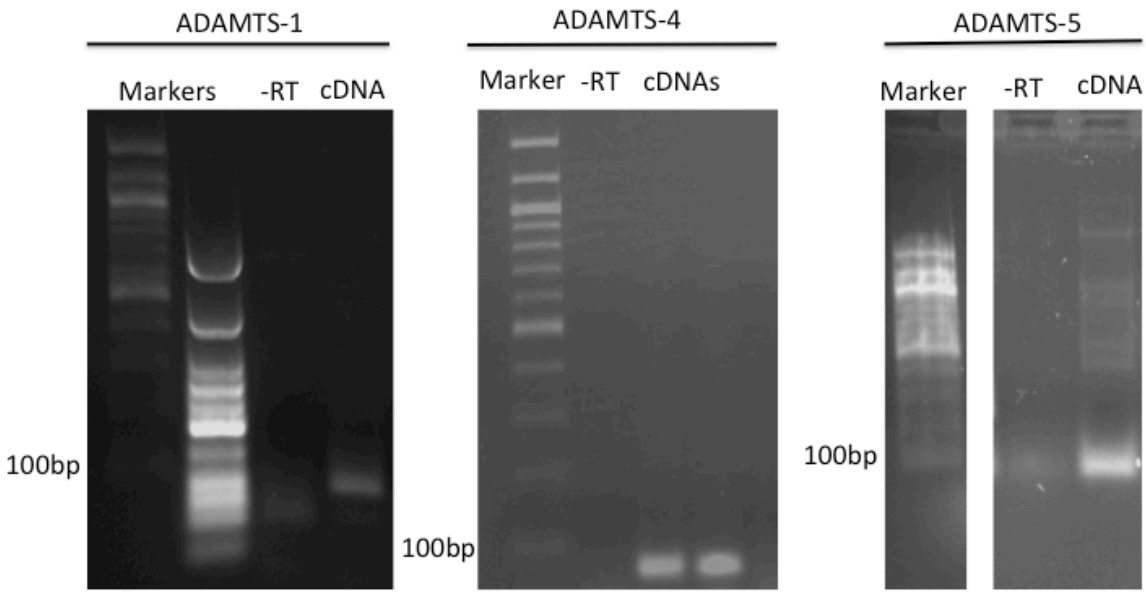


Figure 3.6: ADAMTS-1, -4 and -5 primer set optimisation for Q-PCR

Gel electrophoresis showing the RT-PCR products for ADAMTS-1, -4 and -5 from reactions using their optimised conditions. The amounts of all the PCR reagents were the same in each reaction, but the annealing temperatures were as follows: ADAMTS-1 = 60°C, ADAMTS-4 = 64°C and ADAMTS-5 = 62°C. The size of the expected products were ADAMTS-1 = 90bp, ADAMTS-4 = 81bp and ADAMTS-5 = 93bp. Intermediate not relevant lanes were removed from the ADAMTS-5 image.

3.3.2.2 - Cloning into pGEM-T vectors

After optimisation of the PCR conditions it was important to confirm that the primers were amplifying the correct product. The correct fragment size of the PCR product was confirmed by gel electrophoresis, but as an extra measure the PCR product was cloned into a pGEM-T vector system (Promega) and then sequenced. When the product had been sequenced a BLAST search was carried out to confirm the identity of the gene, the BLAST search did confirm the identity of ADAMTS-1, -4 and -5 PCR products. The alignment of the expected amplicons and the sequencing results are presented in Figure 3.7.

ADAMTS-1 (5'-3')

```
GCACTGCAAGGCGTAGGACAGCCCACAGGAACTGGAAGCATAAGAAAGAAGCGATTTGTGTCCAGTCACCGCTATGTGGAAACCATGCTT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGTGACGTTCCGCATCCTGTCGGGTGTCCTTGACCTTCGTATTCTTTCTTCGCTAAACACAGGTCAGTGGCGATACACCTTTGGTACGAA
```

ADAMTS-4 (5'-3')

```
TCACTGACTTCCTGGACAATGGCTATGGGCACTGTCTCTTAGACAAACCAGAGGCTCCATTGCATCTGCCTGTGACTTTCC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AGTGACTGAAGGACCTGTTACCGATACCCGTGACAGAGAATCTGTTTGGACTCCGAGGTAACGTAGACGGACACTGAAAGG
```

ADAMTS-5 (5'-3')

```
CACTGTGGCTCACGAAATCGGACATTTACTTGGCCTCTCCCATGACGATTCCAAATTCTGTGAAGAGACCTTTGGTTCCACAGAAGATAAGCG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GTGACACCGAGTGCTTTAGCCTGTAAATGAACCGGAGAGGGTACTGGTAAGGTTTAAGACACTTCTCTGGAAACCAAGGTGTCTTCTATTCGC
```

Figure 3.7: ADAMTS-1, -4 and -5 amplicon sequencing

The RT-PCR products of ADAMTS-1, -4 and -5 were sequenced by the Cardiff University DNA sequencing core using M13-Forward sequencing primers. The expected amplicons are presented along with the RT-PCR primer binding regions (red) in the top row of coding for each gene. The sequencing results and how they align are presented in the bottom row (green).

The optimised conditions were then used to perform the Q-PCR assays, the method of analysis that was used to determine the starting amount of DNA during the Q-PCR assays was the ΔΔCT method (Ginzinger 2002). Melt curve analysis was undertaken during the Q-PCR analysis to confirm that the ADAMTS-1, -4 and -5 primers were producing a single, specific product. The melt curve was carried out on the PCR machine after completion of the amplification reaction; increasing the temperature in small increments and monitoring the fluorescent signal at each step generated the melt curve. A plot of the rate of change in fluorescence vs. temperature had distinct peaks that corresponded to the melting temperature of each specific product (Figure 3.8).

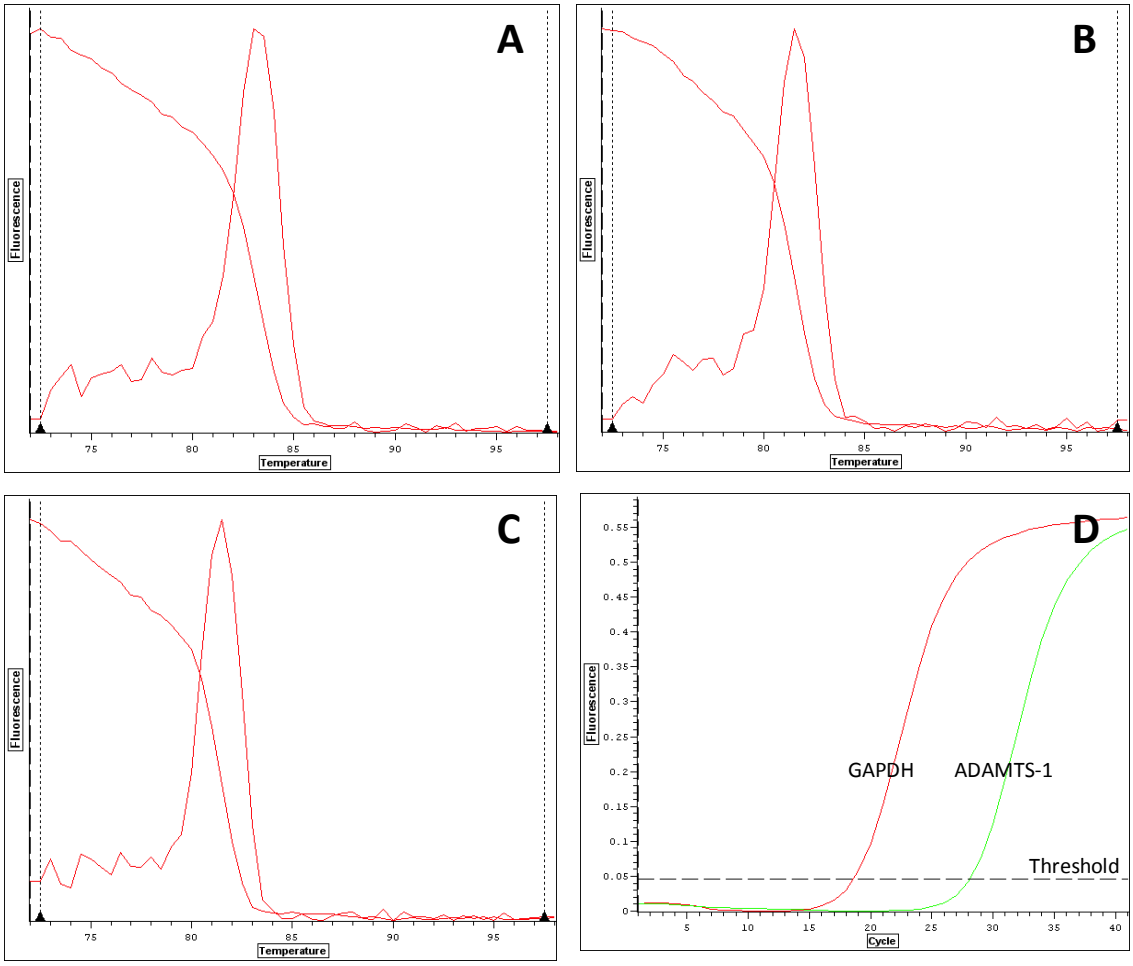


Figure 3.8: **Melt curve analysis during Q-PCR showed that single, specific products were produced from ADAMTS-1, -4 and -5 primer sets**

Melting curve analysis for 3 different primer sets, (A) ADAMTS-1, (B) ADAMTS-4 and (C) ADAMTS-5. Panel (D) shows a representative real time Q-PCR graph displaying two separate reactions carried out with GAPDH (housekeeping) and ADAMTS-1 primer sets. The plots are representative of the data obtained throughout this thesis.

3.3.3 – Expression of ADAMTS-1, -4 and -5 in differentiated THP-1 cells

It was of interest to develop a cellular model of human macrophages that could be used to study ADAMTS-1, -4 and -5 gene expression and regulation. THP-1 cells were the chosen cell line as their responses are conserved between primary macrophages and *in vivo* (Auwerx 1991). The THP-1 cells started out as a monocytic cell line growing in suspension, following addition of PMA they differentiate into macrophages, adhering to

the tissue culture plate. A positive control to ascertain confirmation that PMA was producing its predicted effect upon monocytic THP-1 cells was performed. ApoE and LPL have been shown previously in our laboratory and elsewhere to be induced by PMA stimulation in a time dependant pattern and were therefore used as positive controls (Auwerx et al. 1989; Basheeruddin et al. 1992). The expression of apoE and LPL mRNA after PMA stimulation of THP-1 cells was studied using Q-PCR; the results are displayed in Figure 3.9.

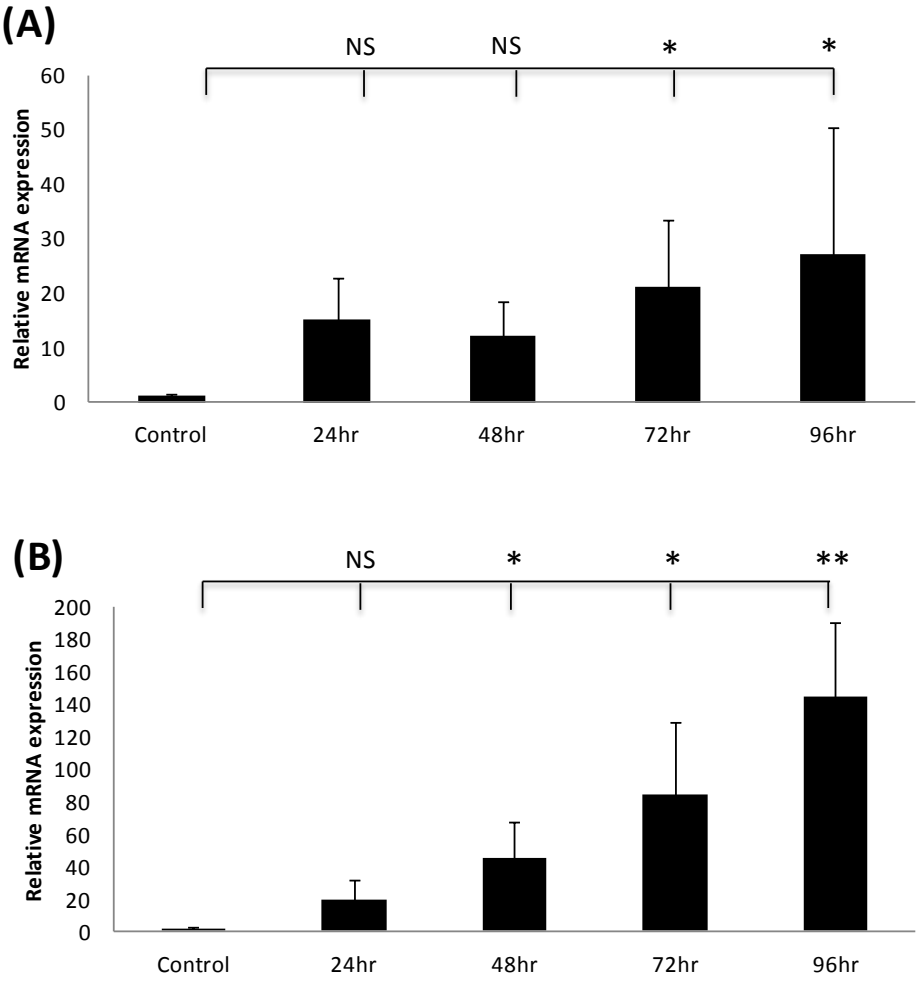


Figure 3.9: ApoE and LPL mRNA expression was induced in differentiated THP-1 cells

Total RNA from untreated (0h - monocytic control) and 0.16µM PMA stimulated (24hr, 48hr, 72hr and 96hr) THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = apoE and B = LPL. The graphs display the average fold change in mRNA expression in relation to the 0h - monocytic control (arbitrarily assigned as 1), from 4 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. *P= <0.05.

As expected apoE and LPL expressions were significantly induced by PMA treatment of THP-1 cells. ApoE expression had some variability, but the general trend towards a time dependent increase after PMA stimulation was observed (Figure 3.9, panel A). LPL data were less variable with a significant increase in mRNA expression being observed after 48hrs of PMA stimulation. The LPL data showed a very clear time dependent increase in mRNA expression after PMA stimulation (Figure 3.9, panel B). Following on from this, the regulation of ADAMTS-1, -4 and -5 expressions in THP-1 cells was studied after PMA stimulation using Q-PCR (Section 2.4.11). The reason a time-course was carried out with PMA was to identify the optimal time that ADAMTS expression was induced during differentiation. Figure 3.10 shows that as expected ADAMTS-1, -4 and -5 mRNAs were expressed in THP-1 monocytes and their differentiation into macrophages using PMA significantly increased their expression over time (Figure 3.10, panels A, B and C). The induction was significant after 24hrs of PMA stimulation in all cases. When the length of PMA stimulation was increased the induction of ADAMTS-1, -4 and -5 mRNA expressions also increased. On average, 96hrs of PMA stimulation increased ADAMTS-1, -4 and -5 mRNA expressions by around 5-10 fold (Figure 3.10, panels A, B and C).

Figure 3.11 shows results from representative experiments that were subjected to western blot analysis and confirms that ADAMTS-4 protein expression was significantly increased after 24hrs and 48hrs of PMA stimulation. Again, due to technical difficulties when using the ADAMTS-1 and -5 antibodies only ADAMTS-4 protein expression could be detected by western blot. From herein only ADAMTS-4 expression will be analysed by SDS-PAGE and western blot. A small amount of variation occurred at 72 and 96hrs but the general trend can still be followed (Figure 3.11). Representative experiments were also carried out to assess the viability of cells using trypan blue exclusion assay and propidium iodide staining (data not shown). These assays showed negligible cell death at early time points. However, a significant, but variable, level of cell death was observed at 96hrs (~25-35%), which could account for the large variation seen for this time point.

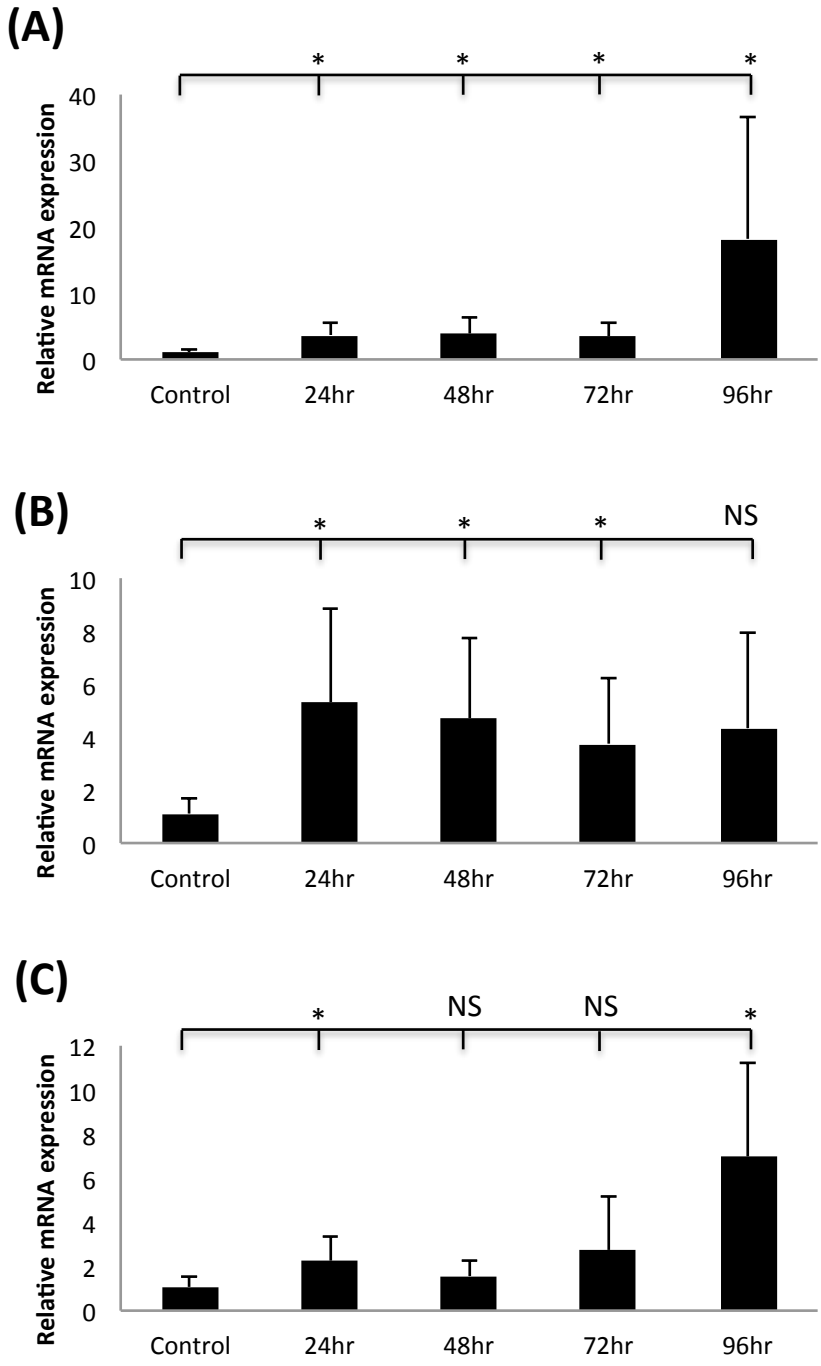


Figure 3.10: ADAMTS-1, -4 and -5 were expressed in differentiated THP-1 cells

Total RNA from untreated (0h – monocytic control) and 0.16µM PMA stimulated (24hr, 48hr, 72hr and 96hr) THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the 0h – monocytic control (arbitrarily assigned as 1), from 7 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. * P= <0.05.

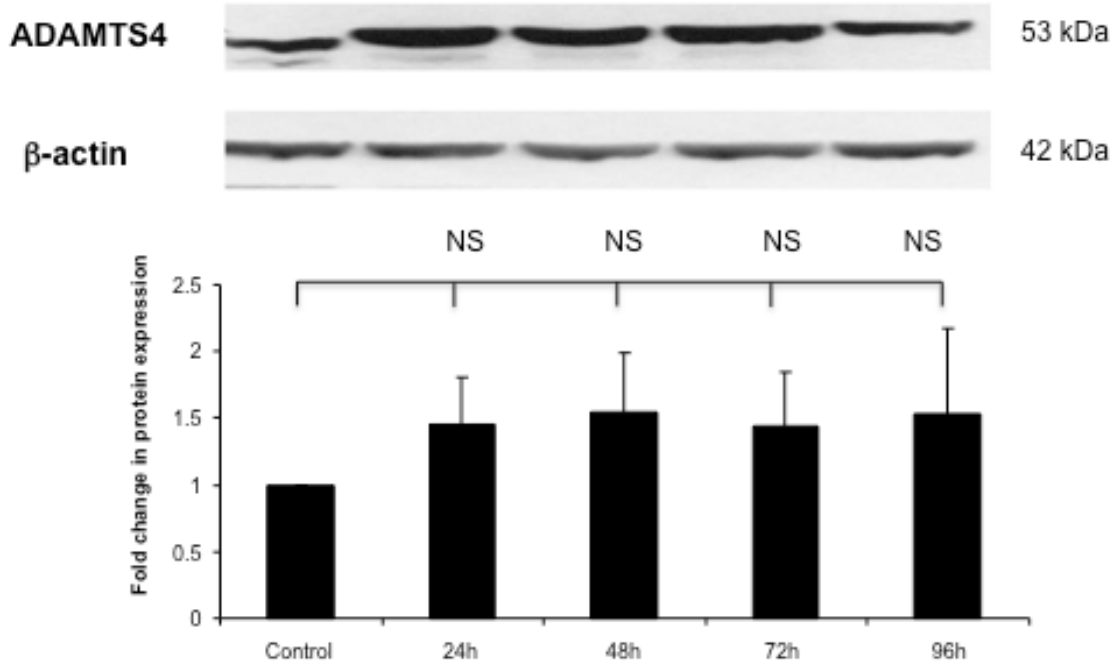


Figure 3.11: ADAMTS-4 protein expression was induced during THP-1 differentiation

Whole cell protein extracts from monocytic THP-1 cells (0h - control) or stimulated (24, 48, 72 or 96hr) with 0.16μM PMA were subjected to SDS-PAGE and western blotting using antibodies against ADAMTS-4 and β-actin. The graph displays the average fold change in protein expression in relation to the monocytic control (arbitrarily assigned as 1), from 3 independent experimental repeats. The blot presented is representative of the 3 experimental repeats. Statistical analysis was performed using one-way ANOVA.

It was decided that 24hr differentiated THP-1 cells would be used as the experimental model for further experiments. The increase in expression was not maximal and therefore any regulation of expression by cytokines, either repression or induction could potentially be detected. Also previous studies have shown that 24hr PMA treatment is sufficient to produce significant expression of macrophage cell markers such as CD36, so we could be confident that the THP-1 cells were indeed behaving as macrophages (Maess et al. 2010). Our laboratory and many others have also used THP-1 cells with 24hr PMA differentiation during other studies into cytokine regulation of macrophage gene expression (Auwerx 1991; McLaren et al. 2010a; McLaren et al. 2010b; Salter et al. 2011; Worley et al. 2003).

3.3.4 - Cytokine Stimulation

3.3.4.1 – TGF-β

A dose of 30ng/ml of TGF-β has been used for previous studies within our laboratory and this was shown to down regulate the expression of ADAMTS-4 expression in THP-1 macrophages (Salter et al. 2011). It has been shown previously in our laboratory and elsewhere that macrophage apoE expression is increased by TGF-β stimulation (Singh and Ramji 2006a; Zuckerman et al. 1992). A positive control experiment was carried out to confirm the recombinant TGF-β was physiologically active. Figure 3.12 shows that apoE expression was significantly increased in THP-1 macrophages after TGF-β stimulation (Figure 3.12, panels A and B). Following on from this the regulation of ADAMTS-1, -4 and -5 expressions was analysed using the above experimental model. The results of the Q-PCR after TGF-β stimulation are presented in Figure 3.13. A range of different TGF-β stimulation time points were tested using SDS-PAGE and western blotting to ascertain the length of time that produced the maximal reduction of ADAMTS-4 expression, the results can be observed in Figure 3.14.

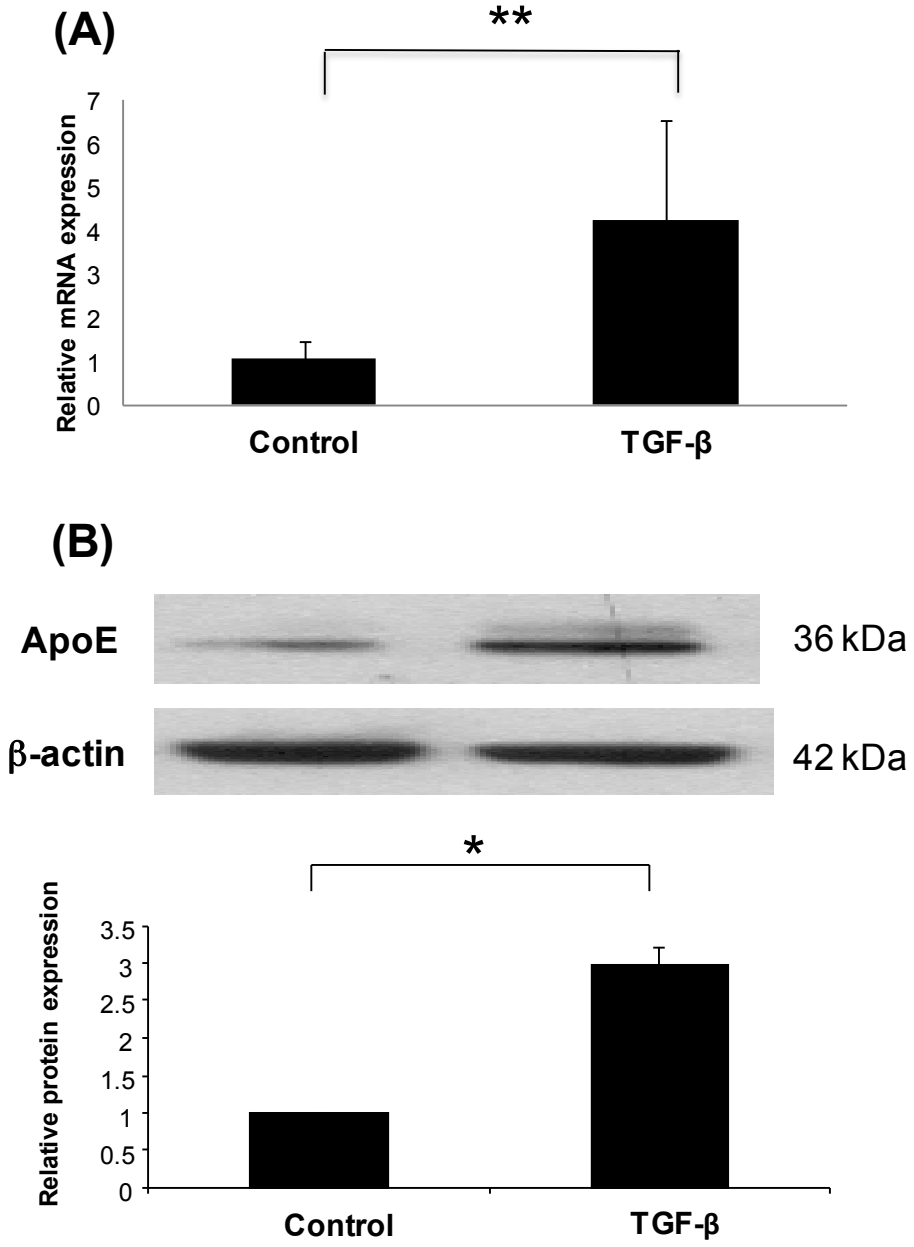


Figure 3.12: ApoE expression was induced after TGF-β stimulation in THP-1 macrophages

24hr differentiated THP-1 cells were either left untreated (control) or stimulated with 30ng/ml TGF-β for 24hrs. In Panel A, total RNA preparations were subjected to a reverse transcriptase reaction and Q-PCR analysis using primers targeting apoE. In Panel B, whole cell protein lysates were subjected to SDS-PAGE and western blots probing for apoE and β-actin. The graphs display the average fold change in mRNA expression and protein expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P= <0.05, **P=<0.01.

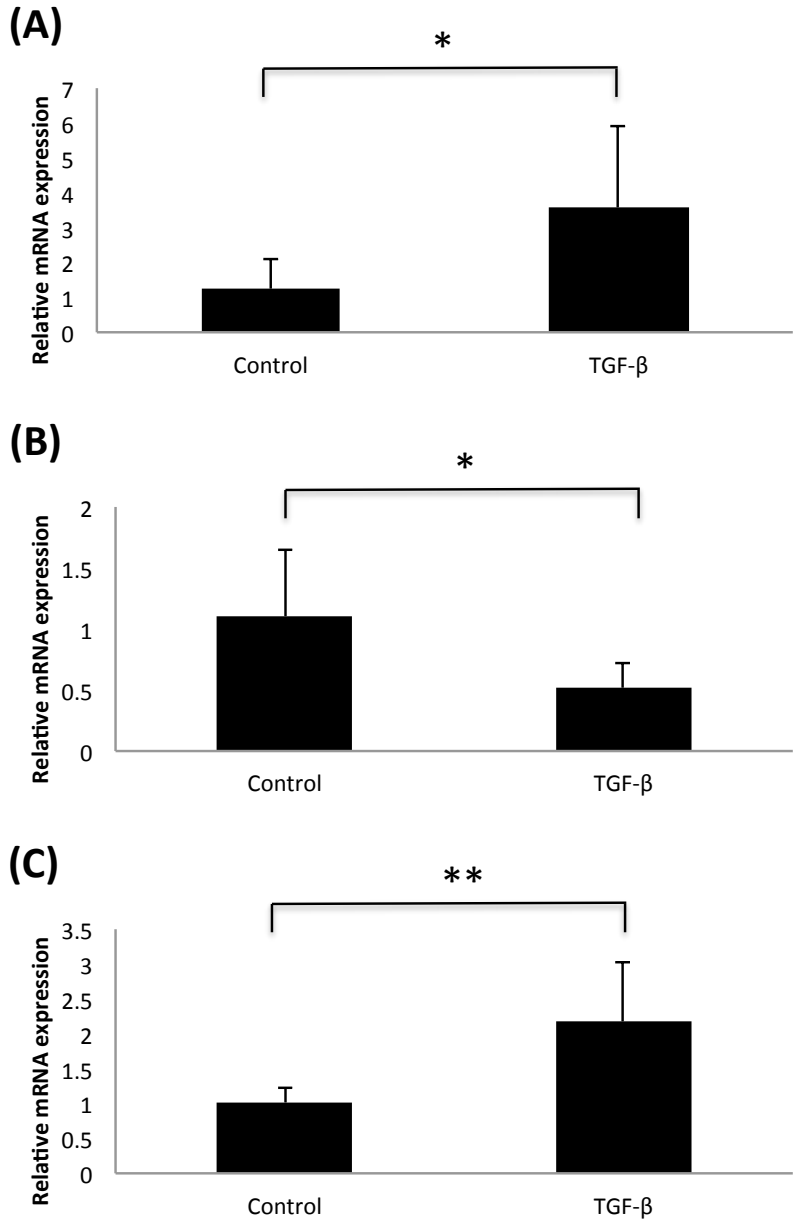


Figure 3.13: ADAMTS-1, -4 and -5 expressions were regulated in THP-1 macrophages after TGF-β stimulation

Total RNA from untreated (control) and 30 ng/ml TGF-β stimulated (24hr) differentiated THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 4 independent experimental repeats. Statistical analysis was performed using the Student's t-test (B and C) or Wilcoxon t-test (A). *P= <0.05, **P=<0.01.

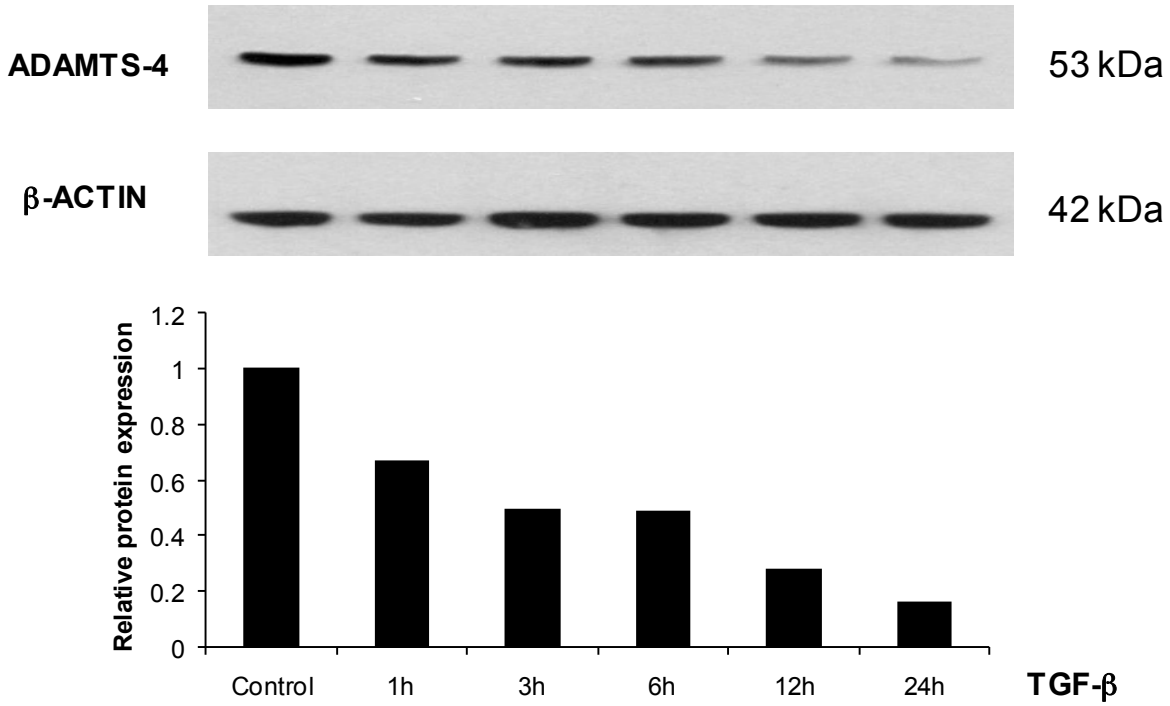


Figure 3.14: ADAMTS-4 protein expression was attenuated after TGF-β stimulation, over time in THP-1 macrophages

Differentiated THP-1 cells (24hr) were either left untreated (0h - control) or stimulated with 30ng/ml TGF-β for 1, 3, 6, 12 or 24hrs. Whole cell protein lysates were subjected to SDS-PAGE and western blots. The graph displays the fold change in protein expression in relation to the control (arbitrarily assigned as 1).

It was observed that ADAMTS-4 mRNA expression was significantly reduced when differentiated THP-1 cells were stimulated with 30ng/ml TGF-β for 24hrs, decreased expression was also observed during SDS-PAGE and western blot analysis (Figure 3.13, panel B and Figure 3.14). These data backs up the findings from a previous study carried out within our laboratory (Salter et al. 2011). In contrast to ADAMTS-4, ADAMTS-1 and -5 mRNA expression was significantly increased after the same TGF-β treatment (Figure 3.13, panel A and C).

3.3.4.2 – IFN-γ

IFN-γ has been widely researched in our laboratory; the concentration that has previously been used is 1000U/ml. The length of stimulation to show regulation has varied in a gene specific pattern, between short response (3hr) and long response (24hr). Taking into

account the length of time that was required during the TGF-β regulation of ADAMTS expression, the 24hr time point was chosen. Similar to the previous section (3.3.4.1) on TGF-β it was of interest to ascertain whether the recombinant IFN-γ being used in the experiments was physiologically active. ApoE and LPL mRNA expression within differentiated THP-1 cells had previously been studied in our laboratory after IFN-γ stimulation (McLaren and Ramji 2009). Due to the pleiotropic role of IFN-γ within atherosclerosis, it has been shown that both apoE and LPL mRNA expression is reduced by IFN-γ stimulation in differentiated THP-1 cells (Brand et al. 1993; Jonasson et al. 1990; McLaren and Ramji 2009). A positive control experiment was carried out to confirm these previous findings; the results are detailed in Figure 3.15. It was observed that apoE and LPL mRNA expression were significantly reduced by IFN-γ stimulation (Figure 3.15, panel A and B). The reduction of apoE protein expression in response to IFN-γ was confirmed by SDS-PAGE and western blot analysis (Figure 3.15, panel C). Following on from the positive control experiment, the regulation that IFN-γ stimulation had on ADAMTS-1, -4 and -5 was assayed using Q-PCR; the results are displayed in Figure 3.16. ADAMTS–1 mRNA expression was significantly reduced by IFN-γ stimulation (Figure 3.16, panel A). ADAMTS-4 and -5 mRNA expressions had no significant change with IFN-γ stimulation (Figure 3.16, panel B and C). These findings differ slightly to that of a previous study (Wagsater et al. 2008). To exclude the possibility that this was not due to the concentration of the cytokine used, a dose response experiment was carried out (Figure 3.17).

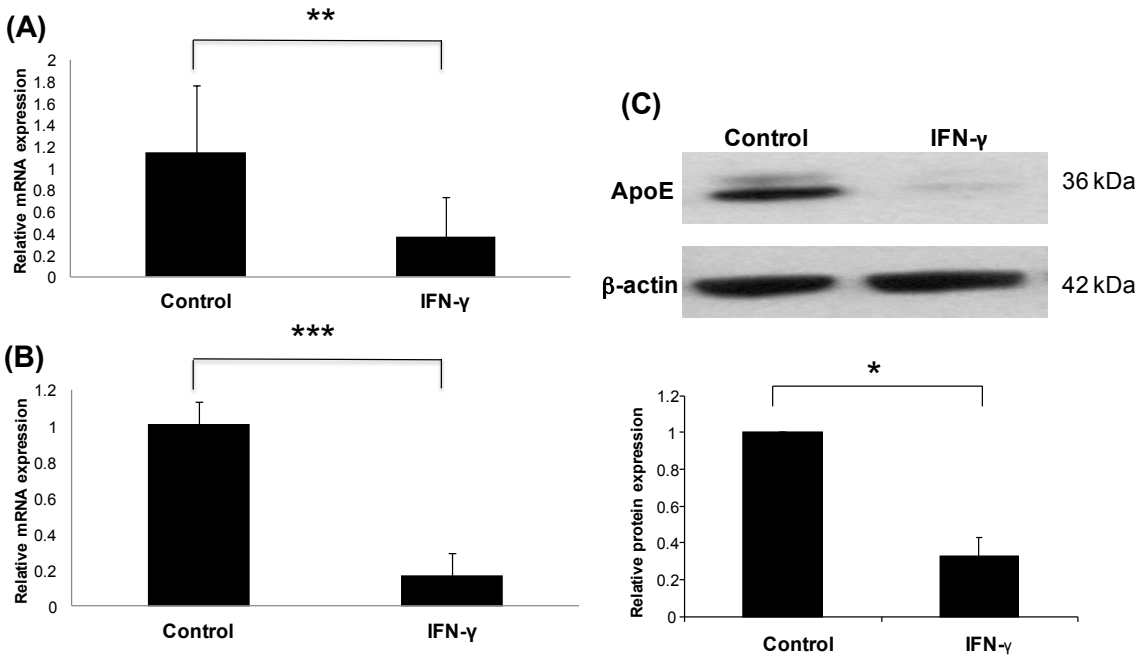


Figure 3.15: Confirmation of IFN-γ attenuation of apoE and LPL expression from differentiated THP-1 cells

The 24hr differentiated THP-1 cells were either left untreated (control) or stimulated with 1000U/ml IFN-γ for 24hrs. In Panel A, total RNA preparations were subjected to a reverse transcriptase reaction and Q-PCR analysis using primers targeting apoE. In Panel B, total RNA preparations were subjected to a reverse transcriptase reaction and Q-PCR analysis using primers targeting LPL. In Panel C, whole cell protein lysates were subjected to SDS-PAGE and western blots probing for apoE and β-actin. The graphs display the average fold change in mRNA expression and protein expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P= <0.05, **P=<0.01 and ***P=<0.001.

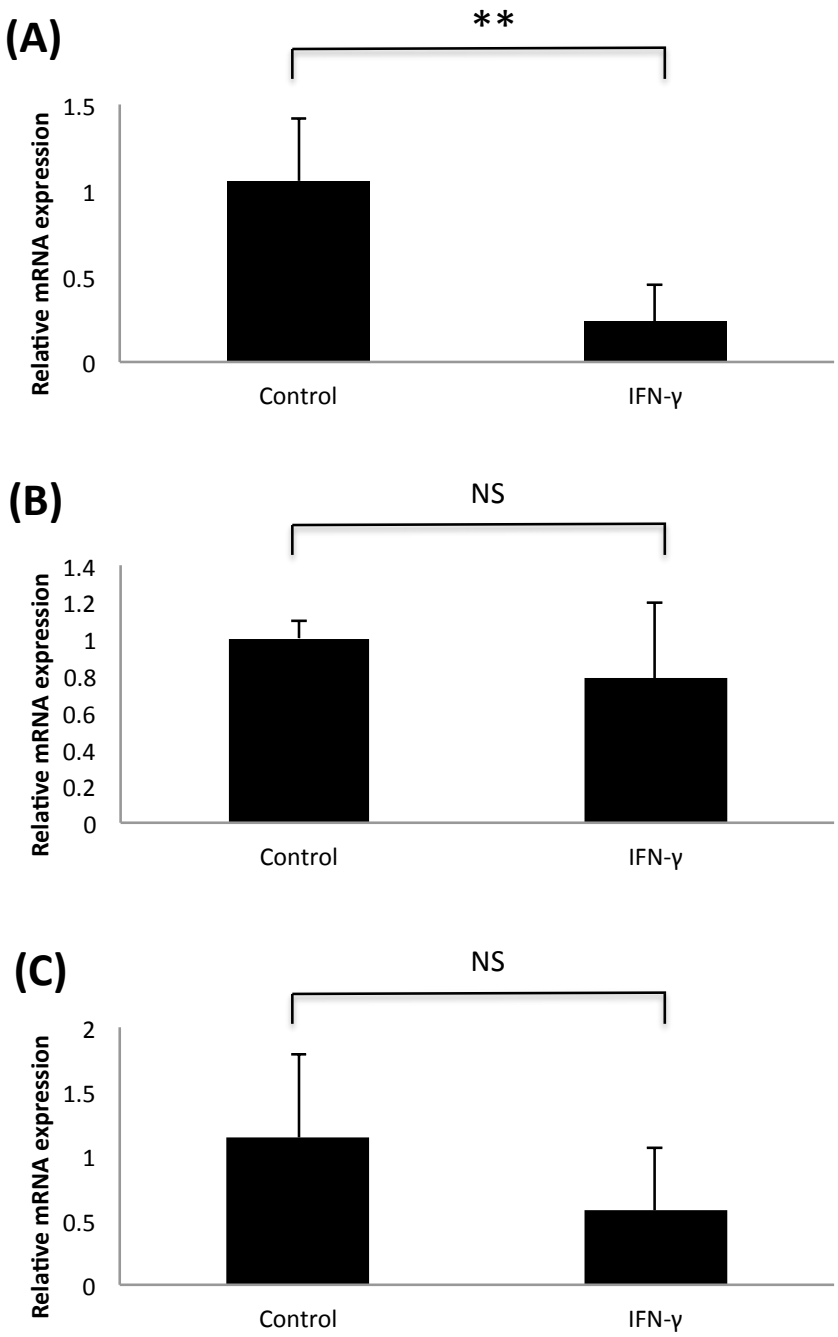


Figure 3.16: IFN-γ stimulation attenuated ADAMTS-1 mRNA expression in THP-1 macrophages

Total RNA from untreated (control) and 1000U/ml IFN-γ stimulated (24hr) differentiated THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test (A) or Wilcoxon t-test (B and C). *P= <0.05, **P=<0.01.

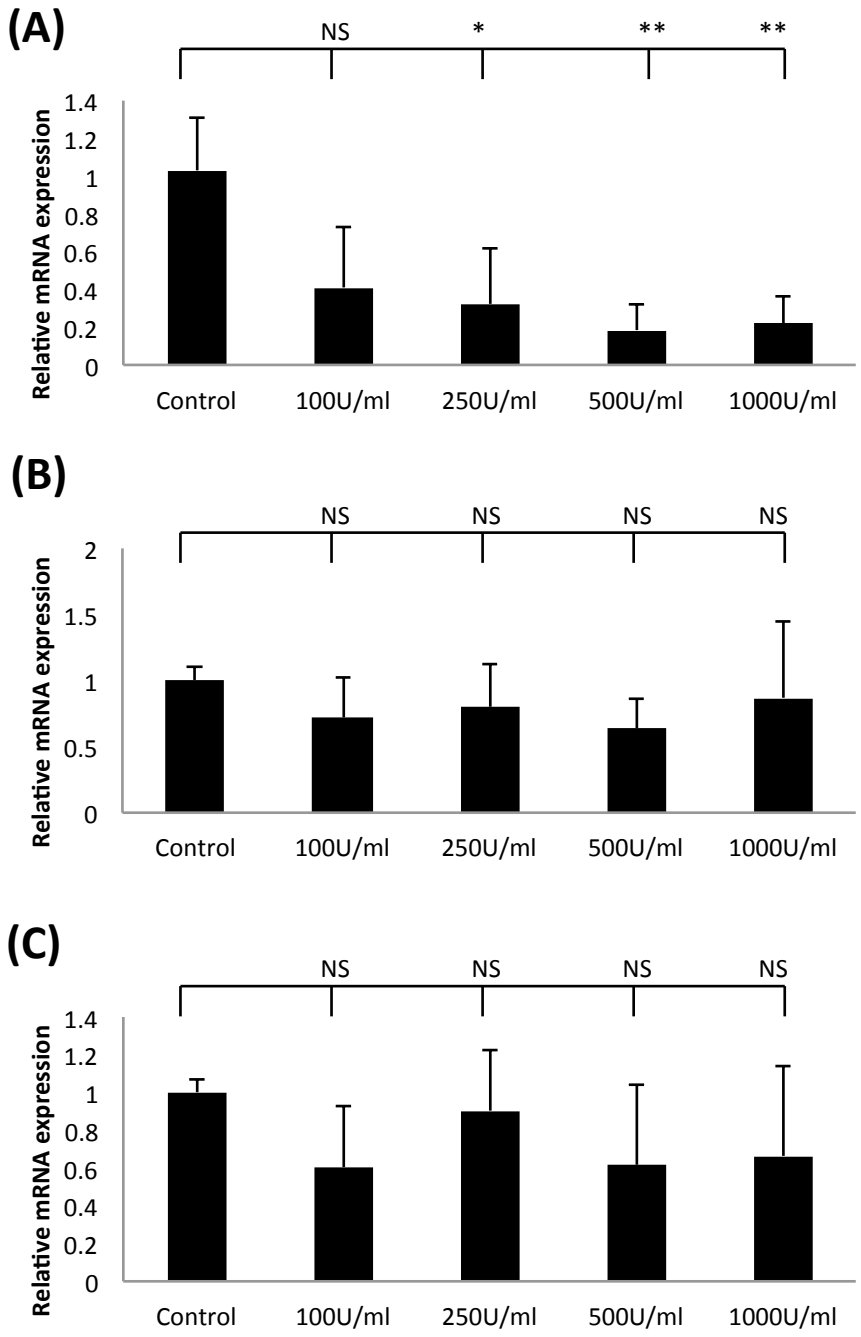


Figure 3.17: IFN-γ stimulation attenuated ADAMTS-1 mRNA in THP-1 macrophages and was dose dependent

Total RNA from untreated (control) and 24hr IFN-γ stimulated (100, 250, 500 and 1000U/ml) differentiated THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. *P= <0.05, **P=<0.01.

After carrying out the IFN-γ dose response, it was confirmed that ADAMTS-1 mRNA expression was reduced by IFN-γ stimulation. A significant reduction in expression was seen with 250U/ml, 500U/ml and 1000U/ml IFN-γ (Figure 3.17, panel A). A concentration of 100U/ml produced a trend towards a reduction in mRNA expression but the reduction was not statistically significant Figure 3.17, panel A). ADAMTS-4 and -5 expressions showed no significant change over all concentrations of IFN-γ tested (Figure 3.17, panels B and C).

After the regulation of ADAMTS-1, -4 and -5 after IFN-γ stimulation had been assayed using differentiated THP-1 cells, representative experiments utilising primary HMDMs were carried out. The results after stimulating HMDMs with 1000U/ml IFN-γ for 24 hours are displayed in Figure 3.18. The findings that had previously been demonstrated in differentiated THP-1 cells were confirmed in HMDM cultures. Only ADAMTS-1 expression was significantly reduced by IFN-γ stimulation (Figure 3.18, panel A). ADAMTS-4 and -5 expressions remained unchanged (Figure 3.18, panels B and C).

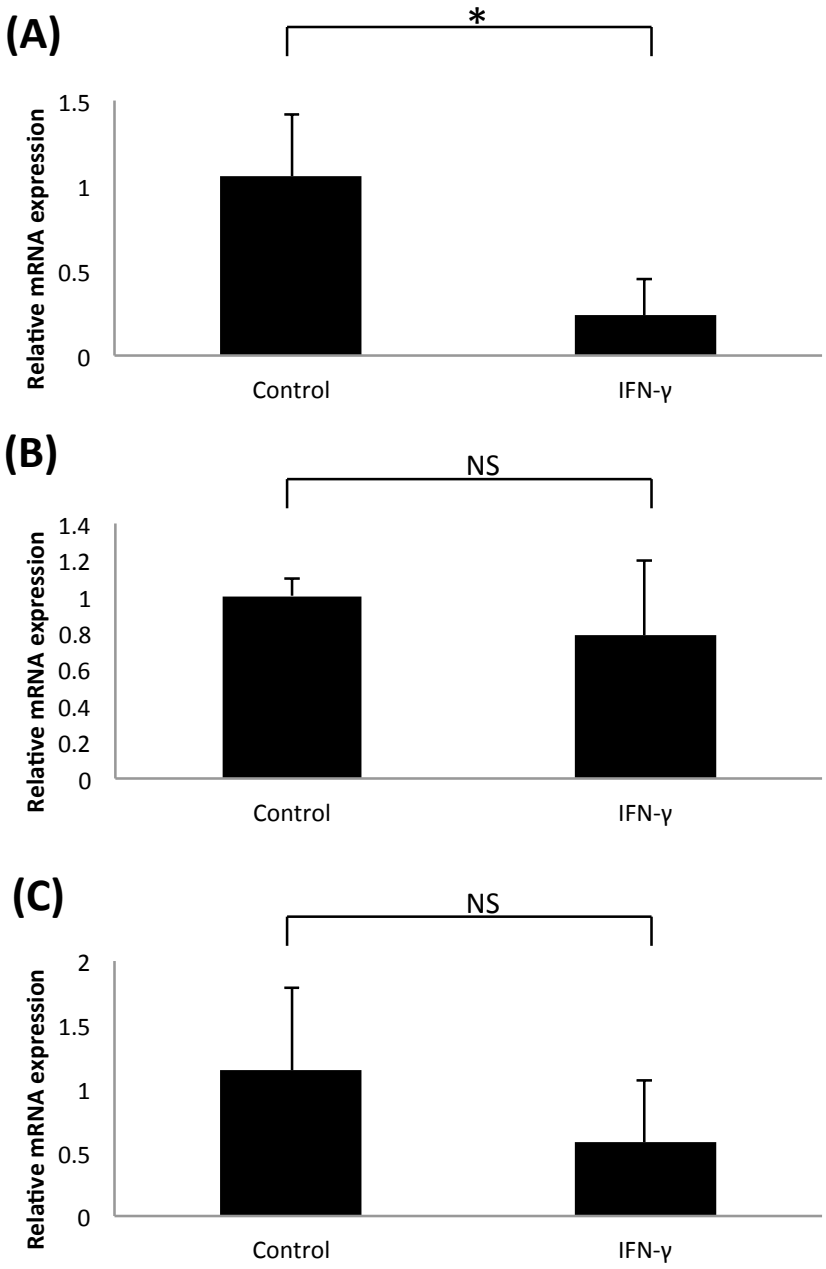


Figure 3.18: IFN-γ stimulation attenuated ADAMTS-1 mRNA expression in HMDMs

Total RNA from untreated (control) and 1000U/ml IFN-γ stimulated (24hr) differentiated HMDMs were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P= <0.05.

3.3.4.3 – TL1A

A previous study from within our laboratory showed that 100ng/ml TL1A stimulation of differentiated THP-1 cells for 24 hours produced a significant reduction in apoE expression (McLaren et al. 2010a). A positive control experiment was carried out using a 100ng/ml stimulation to confirm that the recombinant TL1A was physiologically active. The results are detailed in Figure 3.19. It was shown that apoE mRNA expression was significantly reduced after 100ng/ml TL1A stimulation (Figure 3.19, panel A).

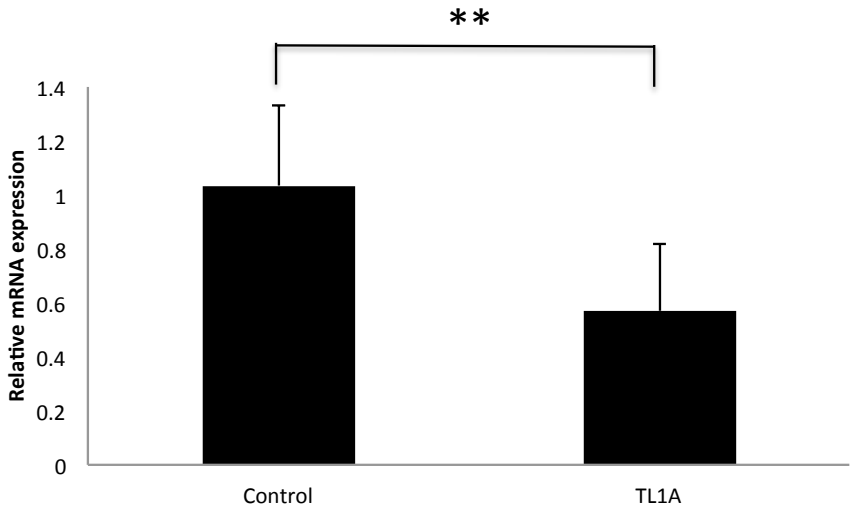


Figure 3.19: ApoE mRNA expression was attenuated after TL1A stimulation in THP-1 macrophages

Total RNA from untreated (control) and TL1A stimulated (100ng/ml) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. The graph displays the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. **P= <0.01.

Following on from the positive control, the results of TL1A regulation over ADAMTS-1, -4 and -5 expressions are presented in Figure 3.20. It was observed that when using 100ng/ml of TL1A no significant change was observed in ADAMTS-1, -4 and -5 expressions (Figure 3.20, panels A, B and C). To investigate whether this was dose dependant we carried out a TL1A dose response on THP-1 cells, the data are presented in Figure 3.21.

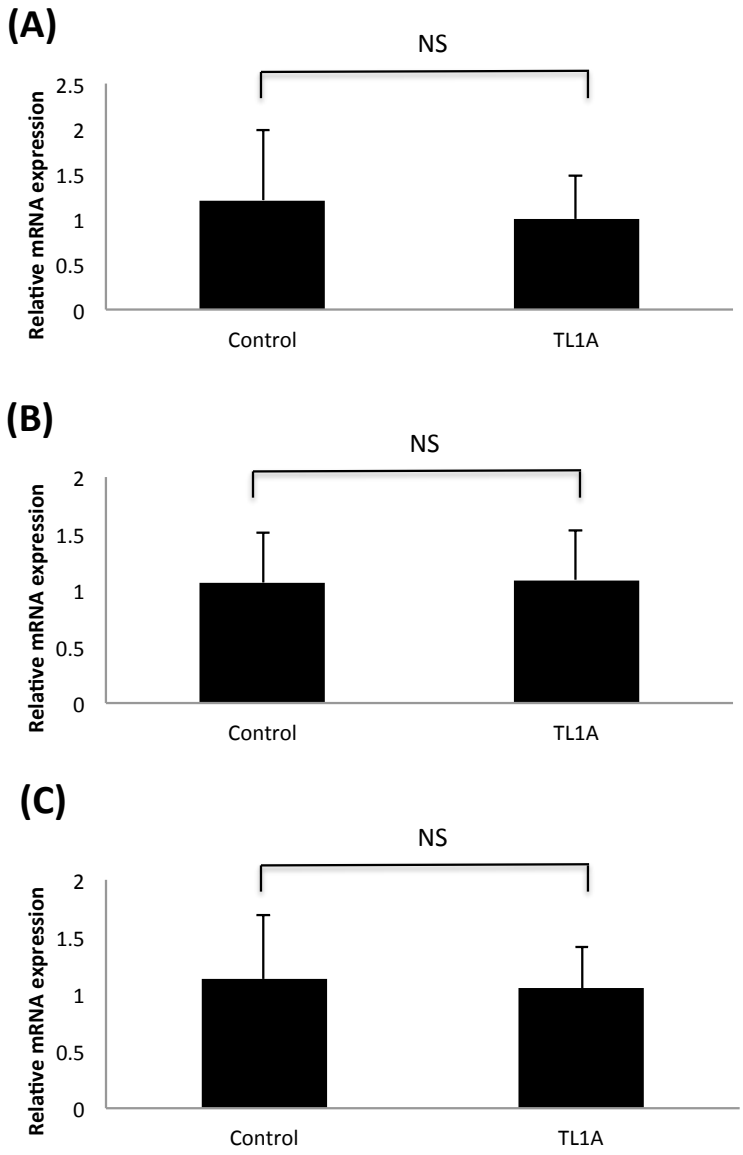


Figure 3.20: ADAMTS-1, -4 and -5 expressions did not change after TL1A stimulation in THP-1 macrophages

Total RNA from untreated (control) and 100ng/ml TL1A stimulated (24hr) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test (A and C) or Wilcoxon t-test (B).

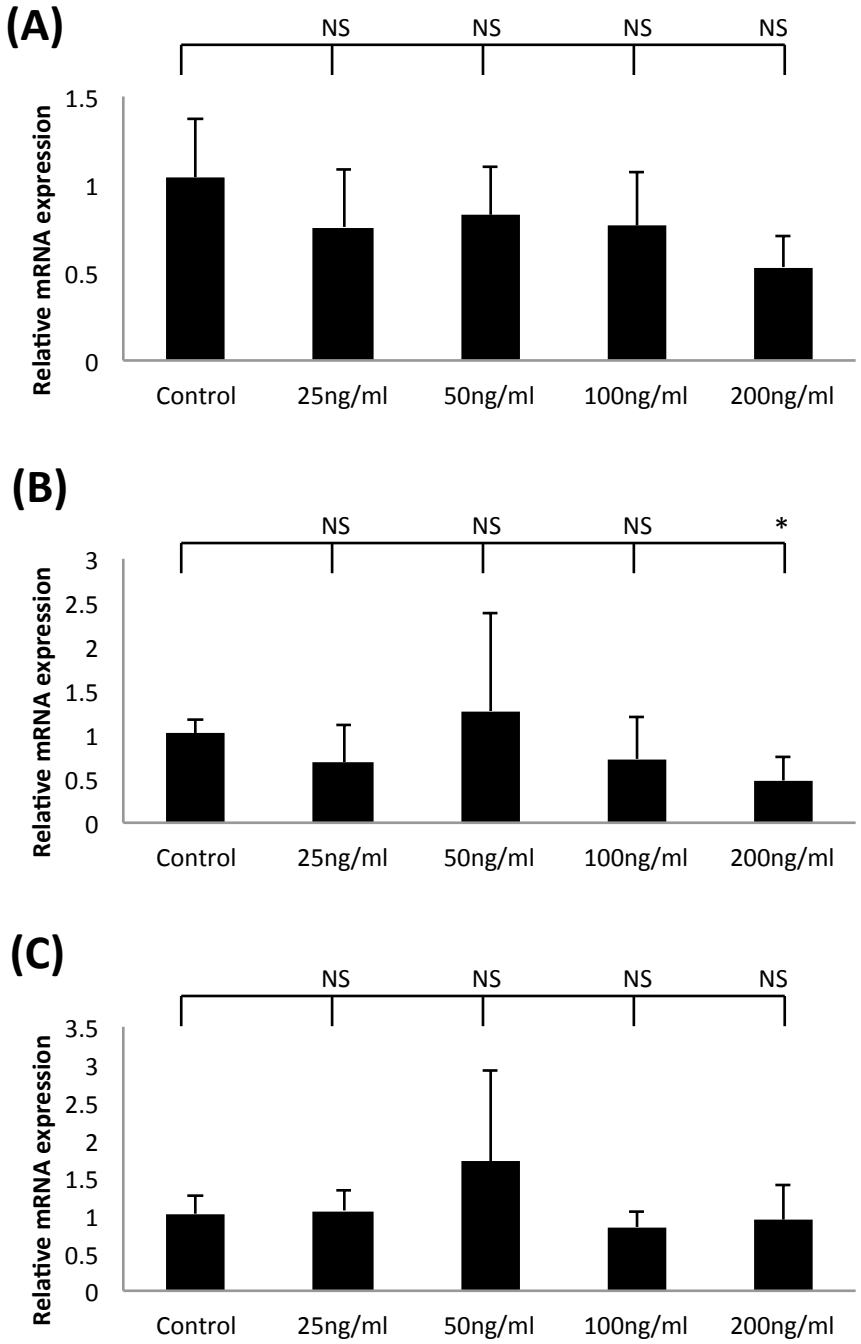


Figure 3.21: ADAMTS-1, -4 and -5 expressions after different doses of TL1A stimulation in THP-1 macrophages

Total RNA from untreated (control) and 24hr TL1A stimulated (25, 50, 100 and 200ng/ml) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. *P= <0.05.

When different doses of TL1A were added to differentiated THP-1 cells it was observed that overall, TL1A stimulation has no significant effect on ADAMTS-1, -4 and -5 expressions (Figure 3.21), and this confirms findings that were initially suggested from Figure 3.21. Only one condition during the study brought about a significant change in ADAMTS-4 expression, this was 200ng/ml of TL1A (Figure 3.21, panel B). Although this is an interesting finding, it was not taken any further as the concentration is very high, and may be outside of the physiological level; a dose this high has not been used in any previous publications where 10ng/ml and 100ng/ml have been used (Bamias et al. 2003; McLaren et al. 2010a).

3.3.4.4 – IL-17A

Doses of IL-17A between 10ng/ml and 100ng/ml have been used during previous published studies (Barin et al. 2011; Shahrara et al. 2009). Based on these previous publications and some other work carried out within our laboratory a concentration of 100ng/ml of IL-17A was chosen for further experiments. A positive control experiment was carried out to confirm that the recombinant IL-17A used was producing results that correlate with data produced from within our laboratory (Figure 3.22). IL-17A has previously been shown to significantly reduce the mRNA expression of apoE. It was observed that 100ng/ml IL-17A stimulation attenuated apoE mRNA expression. Although the data was not quite significant, it was very close (P=0.05008) and therefore it was accepted that IL-17A was producing its expected responses (Figure 3.22).

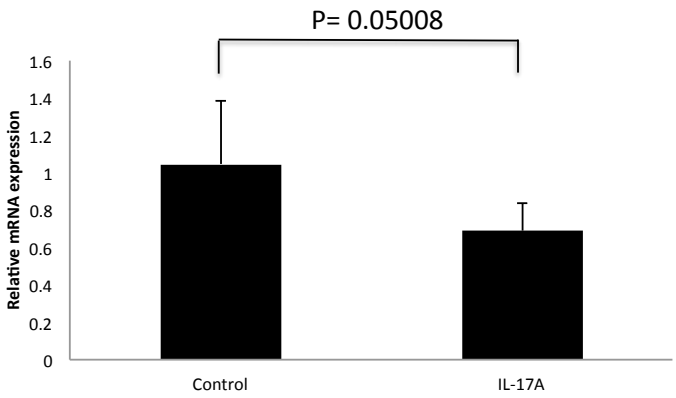


Figure 3.22: ApoE mRNA expression was reduced after IL-17A stimulation in THP-1 macrophages

Total RNA from untreated (control) and IL-17A stimulated (100ng/ml) differentiated THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. The graph displays the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test.

Experiments were carried out to study how IL-17A stimulation of THP-1 macrophages would regulate the mRNA expression of ADAMTS-1, -4 and -5 (Figure 3.23). No significant change was observed in ADAMTS-1, 4 and 5 expressions from differentiated THP-1 cells (Figure 3.23, panels A, B and C). Again, similar to that of the TL1A data presented, we investigated whether this was dose dependant and carried out an IL-17A dose response on differentiated THP-1 cells (Figure 3.24). When different doses of IL-17A were added to differentiated THP-1 cells it was shown that overall, IL-17A stimulation has no significant effect on ADAMTS-1, -4 and -5 expressions (Figure 3.24), and this confirms findings that were initially suggested from Figure 3.24. Only one condition during the study brought about a significant change in ADAMTS-5 expression, this was 200ng/ml of IL-17A (Figure 3.24, panel C). Again, although this is an interesting finding, it was not taken any further as the concentration is very high, and may be outside of the physiological concentration; a dose this high has not been used in any recent previous publications (Barin et al. 2011; Shahrara et al. 2009).

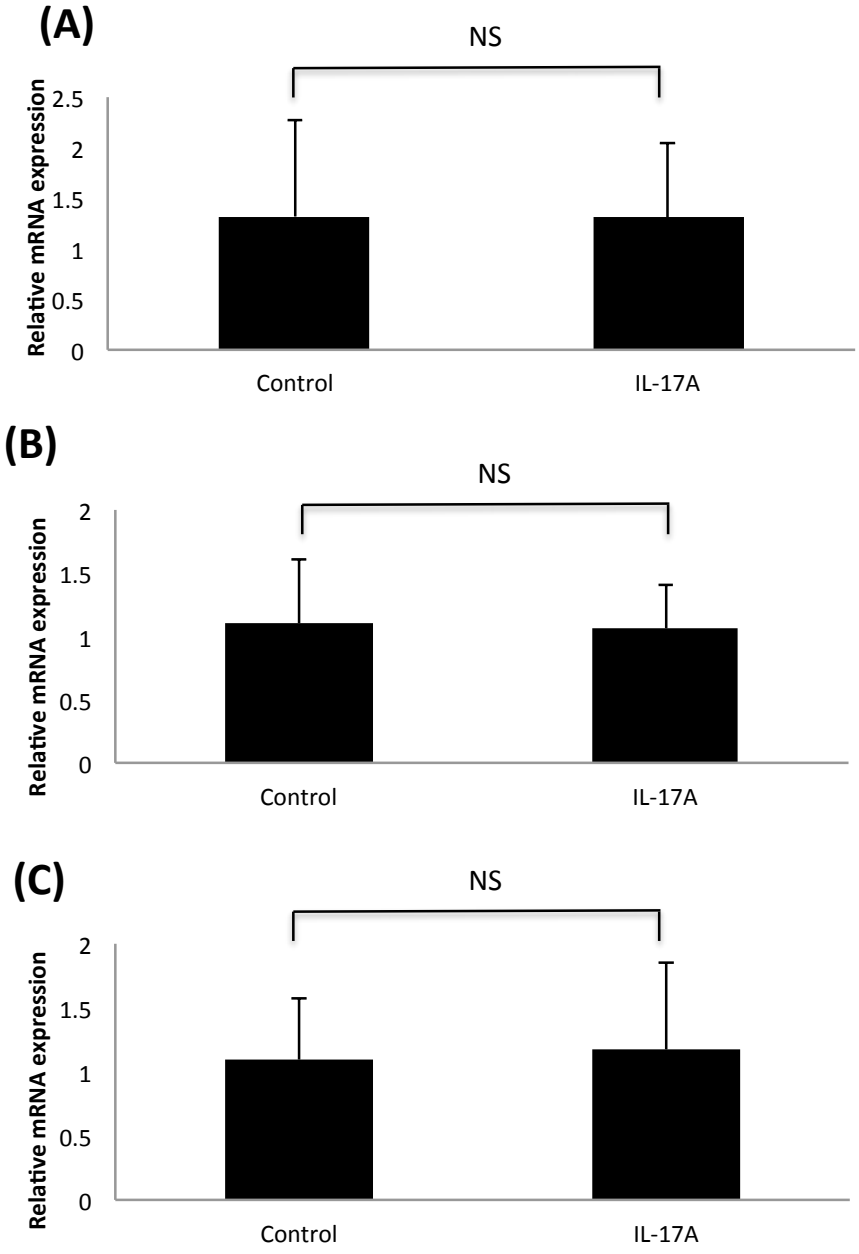


Figure 3.23: ADAMTS-1, -4 and -5 expressions were unchanged after IL-17A stimulation in THP-1 macrophages

Total RNA from untreated (control) and 100ng/ml IL-17A stimulated (24hr) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test.

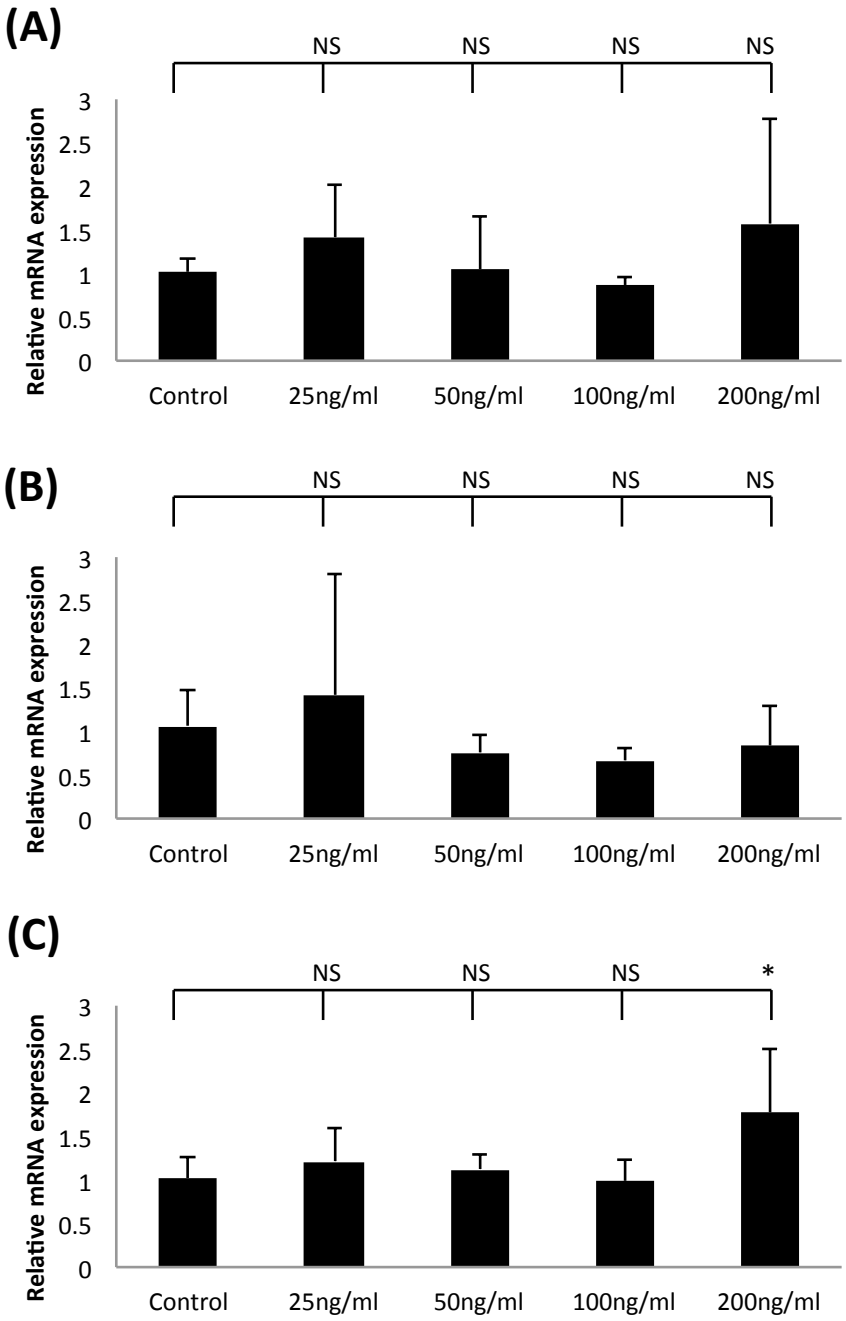


Figure 3.24: ADAMTS-1, -4 and -5 expressions after different doses of IL-17A stimulation in THP-1 macrophages

Total RNA from untreated (control) and 100ng/ml IL-17A stimulated (25, 50, 100 and 200ng/ml) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. *P= <0.05.

3.3.4.5 – IL-33

During a previous published study from within our laboratory a concentration of 10ng/ml IL-33 was shown to reduce macrophage foam cell formation *in vitro (*McLaren et al. 2010b*)*. Based on the ability of this concentration to exhibit macrophage gene regulation, 10ng/ml was chosen for all future experiments. A positive control experiment was carried out to confirm that the recombinant IL-33 was physiologically active (Figure 3.25). It was observed that upon IL-33 stimulation there was a significant increase of apoE mRNA expression. Therefore it was accepted that IL-33 was producing its expected responses (Figure 3.25). It was of interest within this chapter to investigate if IL-33 stimulation of THP-1 cells regulated ADAMTS-1, -4 and -5 expressions (Figure 3.26).

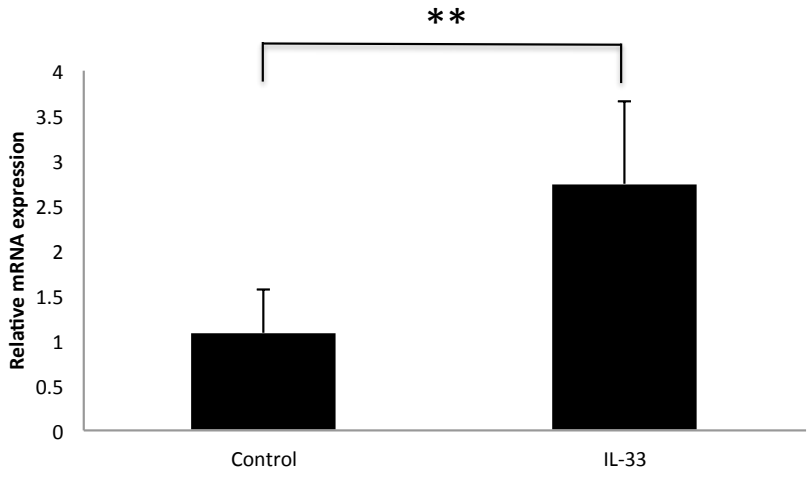


Figure 3.25: ApoE mRNA expression was increased after IL-33 stimulation in THP-1 macrophages

Total RNA from untreated (control) and 10ng/ml IL-33 stimulated (10ng/ml) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. The graph displays the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P=<0.05.

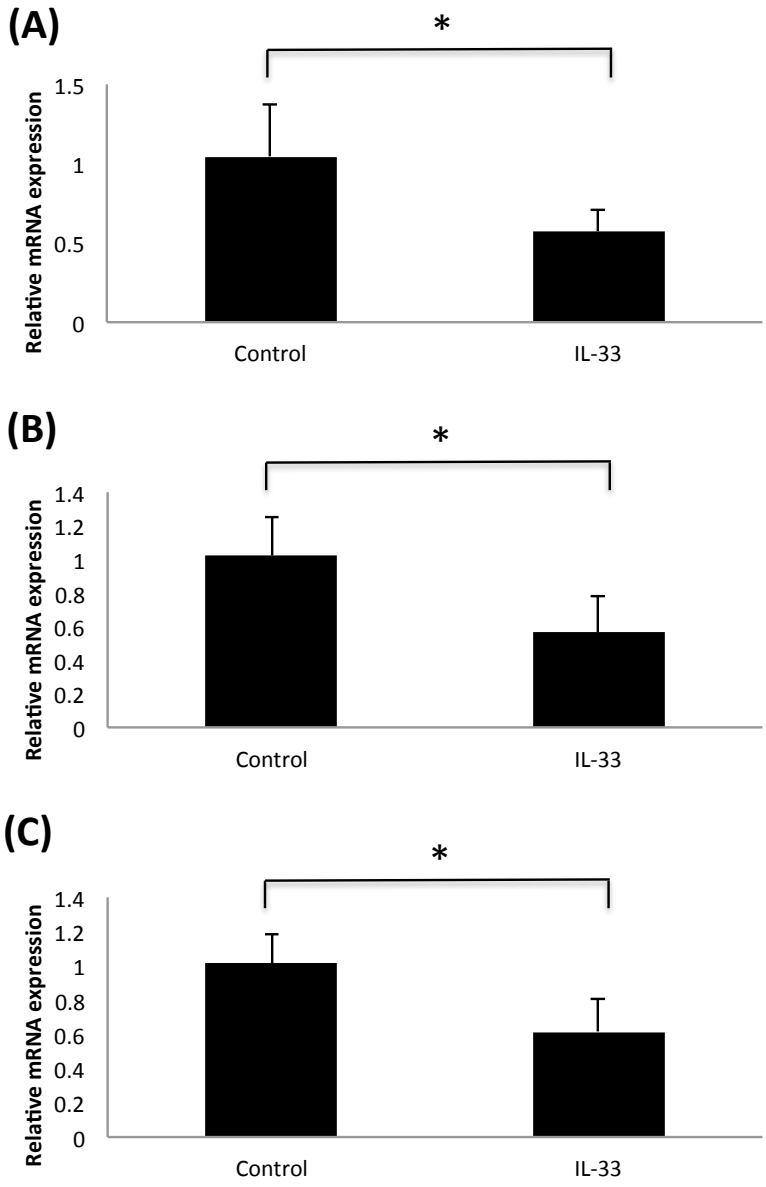


Figure 3.26: ADAMTS-1, -4 and -5 expressions were reduced after IL-33 stimulation in THP-1 macrophages

Total RNA from untreated (control) and 10ng/ml IL-33 stimulated (24hr) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test (A and B) or Wilcoxon t-test (C). *P=<0.05.

It was demonstrated that stimulating differentiated THP-1 cells with 10ng/ml of IL-33 produced a significant reduction in the expressions of ADAMTS-1, -4 and -5 (Figure 3.26, panels A, B and C). ADAMTS-4 expression was confirmed to be reduced by IL-33 stimulation at the protein level utilising SDS-PAGE and western blot analysis (Figure 3.27).

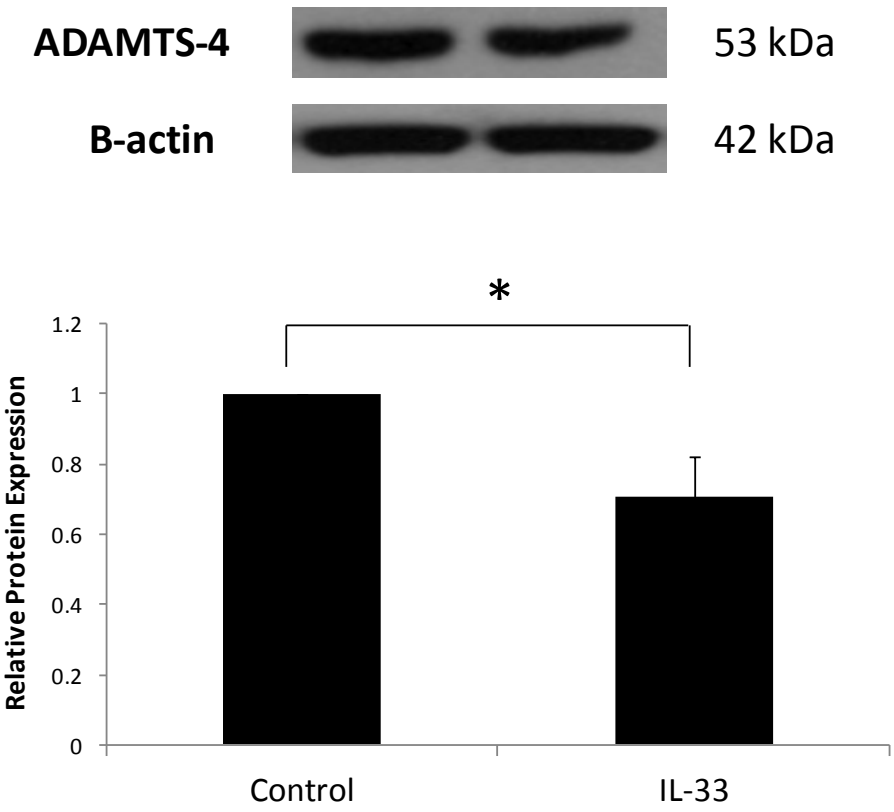


Figure 3.27: ADAMTS-4 protein expression was attenuated by IL-33 stimulation of differentiated THP-1 cells

Whole cell protein extracts from untreated (control) and IL-33 (10ng/ml) stimulated differentiated THP-1 cells were subjected to SDS-PAGE and western blot analysis using antibodies for ADAMTS-4 and β-actin. The graph displays the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P=<0.05.

Representative experiments were carried out utilising IL-33 stimulation on primary HMDMs. The results after stimulating HMDMs with 10ng/ml IL-33 can be observed in Figure 3.28.

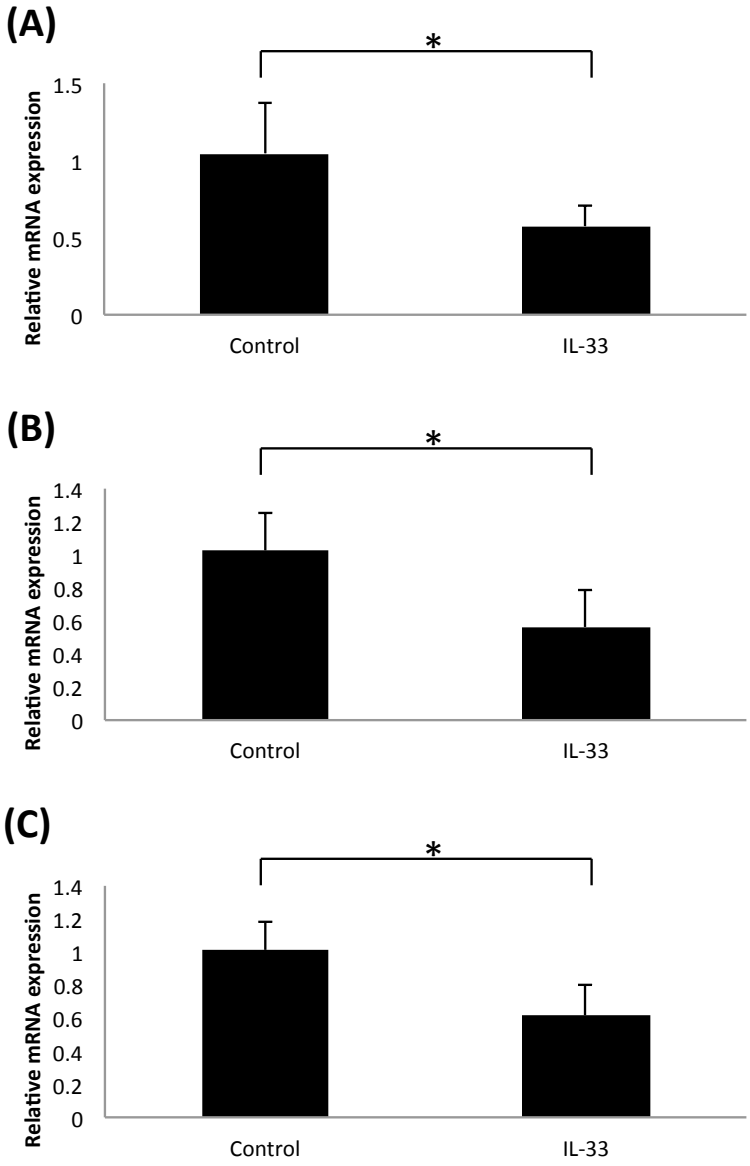


Figure 3.28: ADAMTS-1, -4 and -5 expressions were reduced after IL-33 stimulation in HMDMs

Total RNA from untreated (control) and 10ng/ml IL-33 stimulated (24hr) differentiated HMDMs was subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P= <0.05.

The findings that had been previously demonstrated in differentiated THP-1 cells were confirmed in HMDM cultures. ADAMTS-1, -4 and -5 expressions were significantly reduced by 10ng/ml IL-33 stimulation (Figure 3.28, panel A, B and C).

In order to investigate if IL-33 could also regulate the expression of other proteases from human macrophages, the expression of MMP-9 was studied after stimulation with 10ng/ml IL-33 for 24hrs (Figure 3.29).

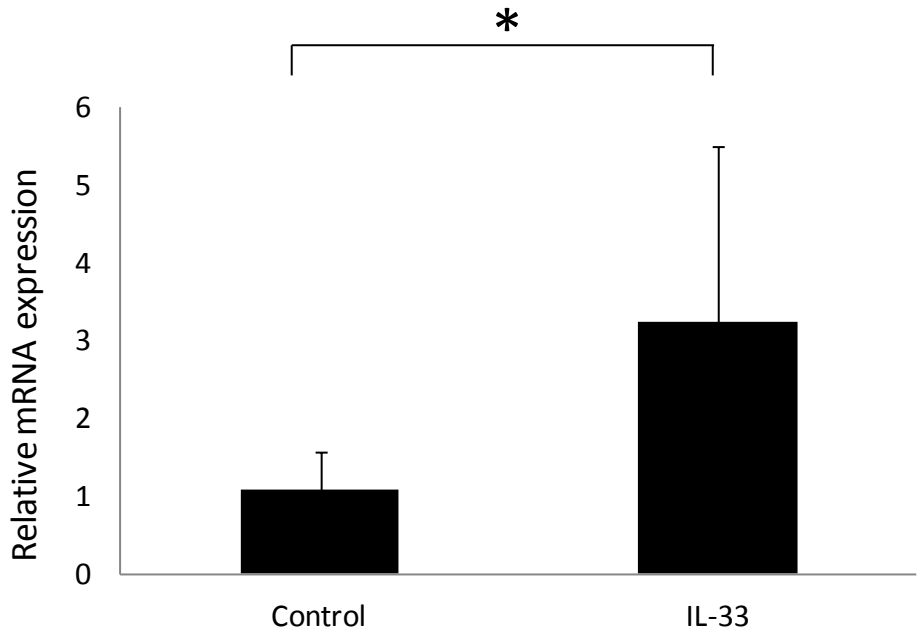


Figure 3.29: MMP-9 expression was induced after IL-33 stimulation in THP-1 cells

Total RNA from untreated (control) and 10ng/ml IL-33 stimulated (24hr) differentiated THP-1 cells was subjected to a reverse transcriptase reaction and Q-PCR analysis. The graph displays the average fold change in MMP-9 mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P= <0.05.

It was demonstrated that stimulating differentiated THP-1 cells with 10ng/ml of IL-33 produced a significant increase in the expression of MMP-9 (Figure 3.29).

3.4 – Discussion

3.4.1 – Summary

Data obtained during this study show that ADAMTS-1, -4 and -5 were expressed mainly by macrophages within human carotid plaques. Following on from the immunohistochemistry, a human cellular macrophage model (THP-1) was developed and characterised. The differentiated THP-1 cells were then stimulated with cytokines that had previously been suggested to act during atherosclerosis. Some cytokines were shown to regulate ADAMTS-1, -4 and -5 gene expressions (Table 3.2).

Table 3.2: ADAMTS-1, -4 and -5 were regulated by cytokines present within the atherosclerotic plaque

	ADAMTS-1	ADAMTS-4	ADAMTS-5
TGF-β	↑	↓	↑
IFN-γ	↓	X	X
TL1A	X	X	X
IL-17	X	X	X
IL-33	↓	↓	↓

↑= Increased expression, ↓= Decreased expression and X= No change in expression

3.4.2 – Immunohistochemistry

3.4.2.1 – Human Carotid Lesions

ADAMTS-1, -4 and -5 expressions were detected in human atherosclerotic plaques (Figure 3.4, panel D, E and F). When looking at the distribution of their expression it was observed that they were mainly expressed by macrophages (Figure 3.4, panel C). A study using immunohistochemistry in 2005 showed that ADAMTS-1 was expressed in human atherosclerotic plaques, with localisation to foam cells and VSMC (Jonsson-Rylander et al. 2005). A further study in 2008 showed that ADAMTS-4 and -5 were present in human atherosclerotic lesions with localisation to CD68 positive macrophage rich regions

(Wagsater et al. 2008). The human immunohistochemical data presented in this chapter confirms findings from these previous publications. Since the immunohistochemical analysis was carried out, an interesting paper has been published. It showed that ADAMTS-1, -4 and -5 were expressed in human atherosclerotic plaques, mainly by CD68 positive macrophages (Lee et al. 2011). ADAMTS-1 expression was elevated within plaques from patients with acute myocardial infarction, verses stable angina; these findings suggest that ADAMTS-1 could have a regulatory role over plaque stability during atherosclerosis (Lee et al. 2011). In separate studies serum levels of ADAMTS-4 have also shown a significant correlation with the severity of coronary artery disease (Chen et al. 2011; Zha et al. 2010b). These findings, taken together, outline the potential regulatory role that ADAMTS proteases could have over the stability of the atherosclerotic plaque.

3.4.3 – Characterising the THP-1 cellular model

Due to the difficulties in consistently accessing human atherosclerotic plaque samples and primary macrophages, the established cell line THP-1 was utilised for the majority of the experiments with key findings confirmed in HMDMs (Kohro et al. 2004). The model has previously been used to study ADAMTS expression from macrophages (Salter et al. 2011; Wagsater et al. 2008; Worley et al. 2003). In this chapter, ADAMTS-1, -4 and -5 expressions were detected from the THP-1 monocytes; 24 hours of PMA stimulation significantly increased their expressions (Figure 3.12 and 13). Previously, a study published in 2003 showed that THP-1 monocytes expressed ADAMTS-4 and upon PMA stimulation, the expression was significantly increased (Worley et al. 2003). The same study also demonstrated that ADAMTS-4 expression was suppressed by anti-atherogenic PPARγ and retinoid X receptor (RXR) agonists (Worley et al. 2003). Another study published in 2008 showed that ADAMTS-1, -4 and -5 were expressed in THP-1 monocytes (Wagsater et al. 2008). After 24hr PMA stimulation ADAMTS-4 expression increased, but ADAMTS-1 and -5 expressions remained unchanged (Wagsater et al. 2008). These findings differ slightly to those obtained within this chapter. One explanation for this inconsistency is that slightly different protocols were used for differentiation of THP-1 cells between the studies. Our study used a system that is employed by majority of the researchers in the field and involves continuous treatment with PMA. On the other hand,

the previous study used techniques that tried to eliminate the direct effect of PMA on ADAMTS expression. They used conditioned media from already differentiated THP-1 cells for differentiation, or they differentiated the cells with PMA for 24hr and then removed the PMA for 24hrs before they began their experiments (Wagsater et al. 2008).

3.4.4 – Cytokine Stimulation

3.4.4.1 – TGF-β

In this chapter, it was observed that ADAMTS-4 mRNA expression was significantly reduced when differentiated THP-1 cells were stimulated with 30ng/ml TGF-β for 24hrs (Figure 3.13, panel B). The reduction in mRNA expression of ADAMTS-4 was also translated into a change in protein level (Figure 3.14). These data back up the findings from a previous study carried out within our laboratory (Salter et al. 2011). ADAMTS-1 and -5 mRNA expressions were significantly increased after the same TGF-β treatment (Figure 3.13, panel A and C). TGF-β has previously been shown to have a protective role during atherosclerosis (Singh and Ramji 2006a). Studies on human and mouse plaques have suggested a plaque-stabilising role for TGF-β, the cytokine acts to lower pro-inflammatory cytokine production, reduce MMP actions and increase collagen synthesis (Bot et al. 2009; Frutkin et al. 2009; Jiang et al. 2004). The ADAMTS-4 data obtained within this chapter is consistent with an anti-atherogenic plaque-stabilising role for TGF-β within atherosclerosis. The ADAMTS-1 and -5 data could suggest that ADAMTS proteases have gene specific regulation and roles within atherosclerotic plaques. The gene specific differences in expression could also be down to the slight pleiotropic regulatory behavior of TGF-β during atherosclerosis (Salter et al. 2011; Singh and Ramji 2006a).

3.4.4.2 – IFN-γ

ADAMTS–1 mRNA expression was significantly reduced by IFN-γ stimulation (Figure 3.18, panel A). ADAMTS-4 and 5 mRNA expressions had no significant change when THP-1 cells were stimulated with IFN-γ (Figure 3.16, panels B and C). A previous publication had already demonstrated that ADAMTS-1 expression was decreased, ADAMTS-4 expression was increased and ADAMTS-5 expression remained the same after IFN-γ stimulation (Wagsater et al. 2008). As previously mentioned, the difference in findings when studying

ADAMTS-4 could be down to the use of a slightly different protocol when differentiating the THP-1 cells. Also, the concentration of IFN-γ used by (Wagsater et al. 2008) was 100U/ml, initially the experiments in this chapter used 1000U/ml. Due to the slight variation in findings a dose response was carried out to try and produce comparative conditions. The dose response revealed concentration of IFN-γ did not alter the responses initially seen when using 1000U/ml (Figure 3.17). The study carried out in 2008 only used differentiated THP-1 cells to study the regulation of ADAMTS expression by IFN-γ (Wagsater et al. 2008). In the present chapter the experiments were extended to HMDMs, therefore the potentially off target effects of PMA were eliminated, the findings remained the same as the THP-1 data (Figure 3.18). IFN-γ has a pro-inflammatory role within atherosclerosis and acts to de-stabilise the plaque, increase MMP production and reduce collagen synthesis (McLaren and Ramji 2009; Nareika et al. 2009; Newby 2007). The results presented in this chapter are not consistent with this pro-inflammatory role of IFN-γ. Previously, ADAMTS-1 has been hypothesised to accelerate plaque progression (Jonsson-Rylander et al. 2005), yet the expression from differentiated THP-1 cells was reduced by IFN-γ. The results presented here highlight the sometimes pleiotropic actions of IFN-γ during inflammation and atherosclerosis (McLaren and Ramji 2009).

3.4.4.3 – TL1A

It was demonstrated that when using 100ng/ml of TL1A, no significant change was observed in ADAMTS-1, -4 and -5 expressions (Figure 3.20, panels A, B and C). This was a surprising result because of the previous evidence of TNF superfamily members being able to regulate ECM degrading proteases (Kang et al. 2005). ADAMTS proteases are closely related to the MMP family of proteases, so it was expected that TL1A could have a regulatory role over ADAMTS-1, -4 and -5 expressions, this was not observed (Salter et al. 2010). To check if this was specific to the concentration used, a dose response experiment was carried out. It was observed that there was no significant change in ADAMTS-1, -4 or -5 expressions after any dose of TL1A (Figure 3.21). Members of the TNF receptor superfamily have previously been implicated in the stimulation of MMP expression (Kang et al. 2005). DR3 is the receptor for TL1A and activation of this receptor has been implicated in the induction of MMP-1, -9 and -13 from THP-1 cells in the presence of IFN-γ (Kang et al. 2005; Kim et al. 2001). These findings, taken with the data

obtained in this chapter, could suggest that DR3 and its ligand, TL1A, have differential actions on different proteases that could influence atherosclerotic plaque stability. Also, TL1A has been shown to have a weaker pro-atherogenic effect when acting on its own. However, co-stimulation with IFN-γ has increased its pro-atherogenic actions (Kang et al. 2005).

3.4.4.4 – IL-17A

It was observed that when using 100ng/ml of IL-17A, no significant change was observed in ADAMTS-1, -4 and -5 expressions from differentiated THP-1 cells (Figure 3.23, panels A, B and C). To check if this was specific to the concentration used, a dose response experiment was carried out. It was observed that there was no significant change in ADAMTS-1, -4 or -5 expressions after any concentration of IL-17A (Figure 3.24). IL-17A and its roles during atherosclerosis are controversial (Danzaki et al. 2012; Usui et al. 2012). The majority of data leads to a pro-atherogenic role but some *in vivo* studies have produced data to challenge this suggestion (Butcher and Galkina 2011). IL-17A is a relatively weak modulator of gene expression; it could however work in combination with other cytokines to produce regulatory effects (Butcher and Galkina 2011). This could explain the variability in some of the *in vivo* data that has been obtained, and also the data that has been obtained during this chapter. Also during this chapter only IL-17A was studied out of the IL-17 family, IL-17E and IL-17F could play a role in regulating atherosclerotic plaque stability as they activate a range of target receptors and signaling pathways and are present within atherosclerotic plaques (de Boer et al. 2009; Moseley et al. 2003).

3.4.4.5 – IL-33

It was demonstrated that stimulating differentiated THP-1 cells with 10ng/ml of IL-33 produced a significant reduction in the expressions of ADAMTS-1, -4 and -5 (Figure 3.26, panels A, B and C). The reduction in ADAMTS-4 expression was confirmed at the protein level utilising SDS-PAGE and western blot analysis (Figure 3.26). Representative experiments were also extended to HMDMs, where reduction in expression of ADAMTS-1, -4 and -5 was also observed (Figure 3.28). IL-33 has been suggested to have a protective role during atherosclerosis development (Miller 2011). The cytokine acts to

promote T_h2 responses, therefore slowing inflammation and stabilising the developing plaque (Miller and Liew 2011). IL-33 has been shown to reduce atherosclerosis in $apoE^{-/-}$ mice being fed a high fat diet (Miller et al. 2008). The data that has been presented in this chapter are consistent with the anti-atherogenic role of the cytokine, IL-33 acts to reduce the expression of ADAMTS proteases which have previously been implicated in ECM remodeling and destabilisation of the atherosclerotic plaque (Salter et al. 2010). It was also demonstrated that IL-33 increased the expression of MMP-9 (Figure 3.29). Normal physiological levels of MMP-9 expression has previously been implicated with a plaque stabilising role during atherosclerosis progression (Newby 2012). These data taken together suggest gene specific regulatory patterns for IL-33 that results in an anti-atherogenic plaque phenotype.

3.4.5 – Future Perspective

The data presented within this chapter proposes that ADAMTS proteases could have an important regulatory role during atherosclerosis. Some cytokines that have been shown to be present within the atherosclerotic plaque have regulatory actions over ADAMTS expression. IL-33 stimulation of macrophages has produced a reduction in the expression of ADAMTS-1, -4 and -5. This response is consistent with the cytokines proposed anti-atherogenic action during atherosclerosis. Important questions that are raised from this data include:

- Can cytokines act in synergy to regulate the expression of ADAMTS proteases?
- What are the intracellular transduction mechanisms utilised to produce the IL-33 regulation over ADAMTS-1, -4 and -5 gene expression?

The next two experimental chapters from this thesis will endeavor to study these important research questions in relation to the role of ADAMTS-1, -4 and -5 in atherosclerosis.

Chapter 4

IL-17A and TL1A can modulate pro-inflammatory responses during co-stimulation

4.1 – Introduction

4.1.1 – IL-17A and Atherosclerosis

T_h17 and IL-17A activity has been linked to the pathogenesis of several autoimmune diseases (Taleb et al. 2010). However, its role in atherosclerosis remains poorly understood and in some cases, controversial (Taleb et al. 2010). The few studies available on this topic have generated contrasting results, which could be attributed to different approaches used on various mouse models (Taleb et al. 2010). Several studies have suggested a pro-atherogenic role of IL-17A through the regulation of macrophage numbers in the plaque, T_h1-related cytokines and chemokine expression (Butcher and Galkina 2011). However, two studies recently described anti-inflammatory effects of IL-17A on mouse plaque burden via possible regulation of aortic VCAM-1 expression and T-cell content (Butcher and Galkina 2011; Cheng et al. 2011; Taleb et al. 2009). To date studies are still producing contrasting conclusions (Butcher et al. 2012; Usui et al. 2012).

The evidence that IL-17A can act to regulate atherosclerosis made it important to study whether it regulates ADAMTS-1, -4 and -5 expressions from human macrophages. In Chapter 3 it was shown that stimulation of differentiated THP-1 macrophages with IL-17A did not regulate the expressions of ADAMTS-1, -4 or -5.

4.1.2 – IL-17A can enhance responses from pro-inflammatory cytokines

IL-17A has been shown to induce the production of pro-inflammatory cytokines from human macrophages (Jovanovic et al. 1998). Co-stimulation of human airway epithelial cells with IL-17A and TNF-α synergistically enhanced the release of IL-8 and chemokine ligand 1 (CXCL1) (Zhu and Qian 2012). IL-17A has been shown to act synergistically with IL-1β to activate the promoter of the CXC chemokine, chemokine-cytokine induced neutrophil chemoattractant (CINC) (Zhu and Qian 2012). IL-17A has also been shown to exacerbate TNF-α-induced oligodendrocyte apoptosis (Paintlia et al. 2011). Recently, a gene array was carried out that investigated the gene regulation of human endothelial cells after co-stimulation with IL-17A and TNF-α (Hot *et al.* 2012). IL-17A alone was able to regulate 248 genes, whereas in combination with TNF-α, 9803 genes were regulated (Hot et al. 2012). Some selected pro-inflammatory responses induced by the synergy were an increase in chemokine ligand 5 (CCL5), IL-8, IL-6 and ICAM-1 expressions (Hot et al. 2012). Epithelial cell migration and invasion were also increased in subsequent studies along with an increased risk of thrombosis (Hot et al. 2012).

Another cytokine that has been shown to behave synergistically with IL-17 is IL-22. Co-stimulation with IL-22 and IL-17A/F resulted in an increased level of antimicrobial peptides to be produced by human primary keratinocytes, showing a regulation of host innate immunity (Liang et al. 2006). IL-17A has also been shown to regulate either the cell-protective or pro-inflammatory actions of IL-22 over epithelial cells. IL-22 acted to protect epithelial cells after bleomycin induced apoptosis, this protection was reversed after addition of IL-17A (Sonnenberg et al. 2010).

IL-17A has also been shown to act in synergy with the pro-inflammatory cytokine IFN-γ (Eid et al. 2009; Zhang et al. 2012). IL-17A significantly enhanced IFN-γ-induced iNOS expression and NO production during *Chlamydia muridarum* infection (Zhang et al. 2012). Also, IFN-γ and IL-17 were shown to be expressed concomitantly by human coronary artery-infiltrating T-cells and acted synergistically to induce VSMC inflammation (Eid et al. 2009).

These data provide evidence to suggest that IL-17A is able to partake in synergistic responses with other pro-inflammatory cytokines. Therefore it was of interest to investigate if IL-17A could act in synergy with other pro-inflammatory mediators to regulate the expression of ADAMTS proteases.

4.1.3 – TL1A

TL1A is the ligand for the DR3 receptor and is a characterized member of the tumor necrosis factor superfamily (Hsu and Viney 2011). Previously, in combination with IFN-γ, DR3 has been shown to have a role in atherosclerosis through stimulation of matrix degrading enzymes including MMP-9, this could lead to de-stabilisation of the plaque (Kang et al. 2005). Expression of TL1A has been shown to be high in regions rich in macrophage/foam cells by immunohistochemical analysis (Kang et al. 2005). These data suggest that TL1A and DR3 are involved in atherosclerosis via the induction of pro-inflammatory cytokines and also by inducing ECM degrading enzymes to reduce plaque stability (Kang et al. 2005). The ability of TL1A to promote the expression of matrix degrading enzymes made it important to study whether it regulates ADAMTS-1, -4 and -5 expressions from human macrophages. In Chapter 3 it was shown that stimulation of differentiated THP-1 macrophages with TL1A did not regulate the expressions of ADAMTS-1, -4 or -5. A pro-atherogenic role for TL1A has been shown in relation to macrophage foam cell formation (McLaren et al. 2010a). The study demonstrated that TL1A promotes foam cell formation in human macrophages *in vitro* by increasing both acetylated and oxidised LDL uptake, by enhancing intracellular, total and esterified cholesterol levels and reducing cholesterol efflux (McLaren et al. 2010a). This imbalance in cholesterol homeostasis was demonstrated to be orchestrated by TL1A-mediated changes in the mRNA and protein expression of several genes implicated in the uptake and efflux of cholesterol, such as SR-A and ABCA1 (McLaren et al. 2010a).

4.1.4 – TL1A can enhance responses from inflammatory cytokines

TL1A, like IL-17A, has been implicated in modulating pro-inflammatory responses from other cytokines. TL1A has been shown to synergise with IL-12 and IL-18 to enhance the

production of IFN-γ from T-cells and NK cells (Papadakis et al. 2004). The synergy between the same agents was also observed when TL1A augmented the IL-12/IL-18-induced IFN-γ production from $CCR9^{+}CD4^{+}$PB T-cells (Papadakis et al. 2005). TL1A synergy was also observed with IL-23, the synergistic response caused an increase in the production of IFN-γ and IL-17A by lamina propria macrophages (Kamada et al. 2010).

A study that has more relevance to atherosclerosis and the role that ADAMTS proteases play in this disease looked at how TL1A synergises with IFN-γ to produce various pro-inflammatory responses from differentiated THP-1 cells (Kang et al. 2005). Anti-DR3 monoclonal antibody treatment of THP-1 cells in combination with IFN-γ produced an induction of TNF-α, MCP-1 and IL-8 productions (Kang et al. 2005). Also, when THP-1 cells were stimulated with TL1A and IFN-γ the expression of MMP-9 and IL-8 were synergistically increased (Kang et al. 2005).

4.1.5 – Synergistic regulation of ADAMTS proteases

In Chapter 3 the regulation of ADAMTS protease expressions by various cytokines was investigated. The studies carried out during Chapter 3 investigated a small group of selected cytokines that were chosen because they have important roles during atherosclerotic progression. IFN-γ, TGF-β and IL-33 were shown to regulate the expressions of ADAMTS-1, -4 and -5 after sole stimulation. IL-17A and TL1A did not exhibit any regulation over ADAMTS-1, -4 and -5 expressions after sole stimulation. Also, some other cytokines have been shown to regulate ADAMTS expressions in some previous studies, including TNF-α (Porter et al. 2005; Salter et al. 2010).

Some previous studies have investigated whether ADAMTS expression can be regulated by co-stimulation with cytokines, these studies have produced some interesting results. In 2005, it was shown that ADAMTS-9 expression was synergistically induced by IL-1β and TNF-α in OUMS-27 chrondrosarcoma cells and human chrondrocytes (Demircan et al. 2005). Another study found that oncostatin M and TNF-α was able to produce a synergistic response and induce the expression of ADAMTS-1, -4, -5 and -9 in bovine chrondrocytes (Hui et al. 2005). When the experiments were repeated in human

chrondrocytes the synergistic response was limited to ADAMTS-1 and -4 (Hui et al. 2005). Finally in 2008, the regulation of ADAMTS-4 from human achilles tendon and tendon-derived cells was studied after co-stimulation with TGF-β and IL-1β (Corps et al. 2008). After 6 hours of co-stimulation a synergistic induction of ADAMTS-4 expression was observed (Corps et al. 2008).

These studies provide evidence that multiple cytokines are able to act synergistically to regulate the expression of ADAMTS proteases. In previous studies it has been shown that TL1A and IL-17A can act in synergy with other pro-inflammatory cytokines such as IFN-γ and TNF-α to enhance their response. However, it has never been studied whether TL1A and IL-17A can act in synergy together to modulate pro-inflammatory responses. Therefore, it was of interest to investigate if the agents could regulate ADAMTS-1, -4 or -5 expressions from macrophages in a synergistic manner, together, or in combination with IFN-γ.

4.2 – Experimental Aims

The primary aim of this experimental chapter was to investigate if IL-17A and TL1A could act in synergy with the pro-inflammatory cytokine IFN-γ, or together, to regulate the expression of ADAMTS-1, -4 and -5 from human macrophages. The synergistic reactions studied were as follows:

- IFN-γ and TL1A
- IFN-γ and IL-17A
- TL1A and IL-17A

The cytokines that were chosen during the investigation were selected based on the literature that had been reviewed in Section 4.1. IL-17A, TL1A and IFN-γ have all been suggested to have acted in synergy during previous studies (Section 4.1). The synergistic responses that had been observed in the previous studies all enhanced the action of pro-

inflammatory cytokines (Section 4.1). Therefore it was of interest to investigate if TL1A, IL-17A and IFN-γ could act together to regulate the expression of ADAMTS-1, -4 or -5.

Firstly, the differentiated THP-1 cell model was utilised for the cytokine stimulation experiments. The cells were co-stimulated with cytokines for 24hrs and the resulting RNA was extracted and analysed using Q-PCR. Any findings were then confirmed by carrying out representative experiments using primary HMDMs.

Finally, after showing a synergistic action between IL-17A and TL1A, it was investigated if the combination could regulate other aspects of macrophage cellular biology in relation to atherosclerosis. A macrophage cholesterol uptake assay was utilised to investigate if the two cytokines could synergise and regulate the cellular uptake of acetylated LDL.

The experimental strategies that were used during this chapter to accomplish these three major aims are outlined in Figures 4.1 – 4.2.

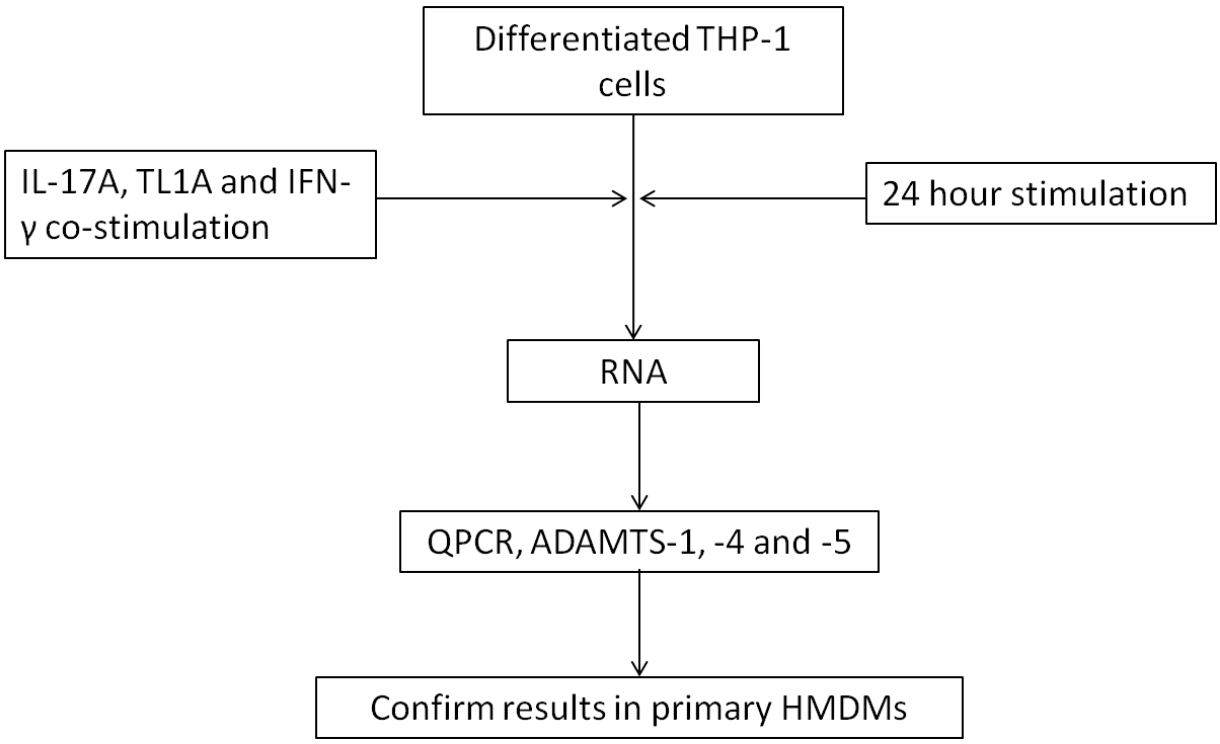


Figure 4.1: **QPCR experimental strategy**

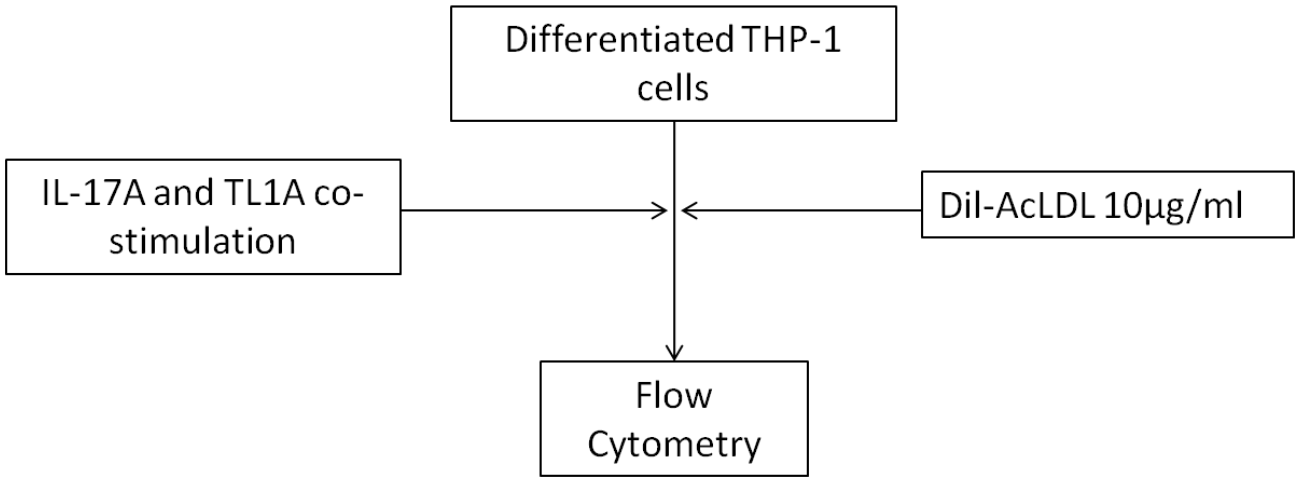


Figure 4.2: **Cholesterol uptake assay strategy**

4.3 – Results

4.3.1 – Cytokine stimulation of THP-1 cells

4.3.1.1 – TL1A and IFN-γ

In order to investigate if TL1A could act in synergy with IFN-γ to regulate ADAMTS-1, -4 or -5 expressions, differentiated THP-1 cells were stimulated with either 100ng/ml TL1A or 1000U/ml IFN-γ on their own, or co-stimulated with both together. The resulting total RNA was analysed by QPCR to study ADAMTS-1, -4 and -5 gene expressions (Figure 4.3).

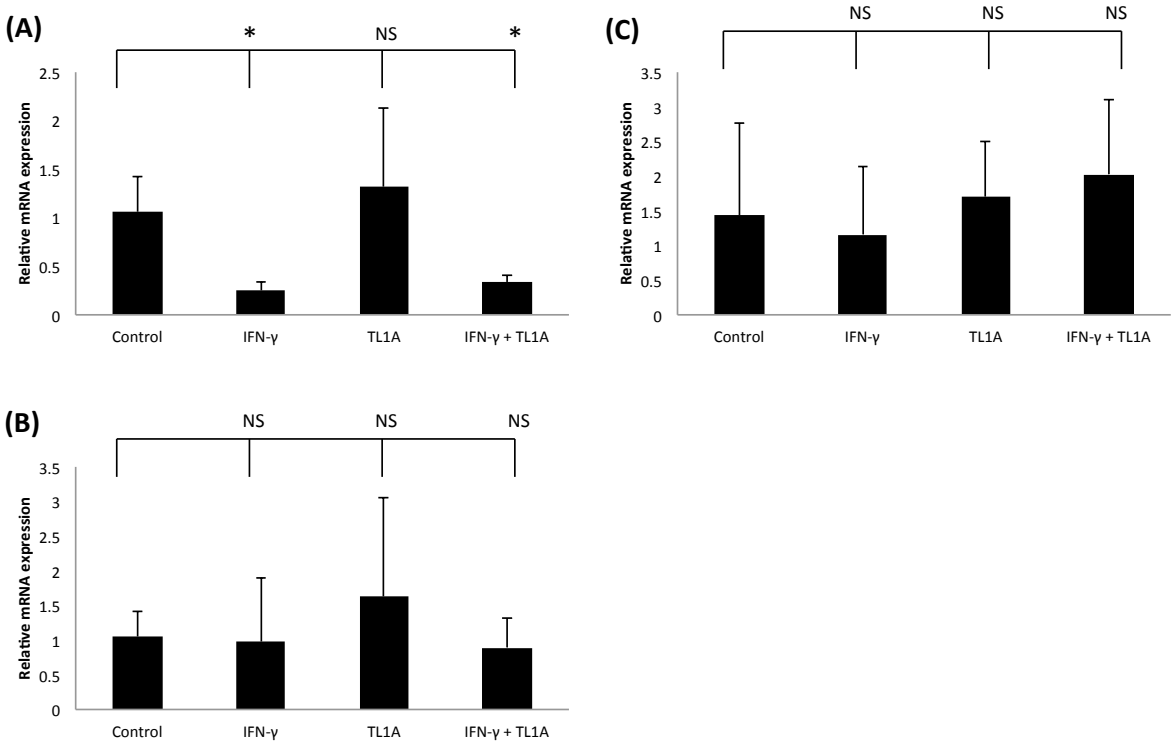


Figure 4.3: TL1A did not synergise with IFN-γ to regulate the expression of ADAMTS-1, -4 or -5 in differentiated THP-1 cells

Total RNA from untreated (control) and 24hr cytokine stimulated (100ng/ml TL1A and 1000U/ml IFN-γ) differentiated THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. * P= <0.05.

Figure 4.3 shows that when differentiated THP-1 cells were stimulated with IFN-γ, ADAMTS-1 expression was significantly reduced and ADAMTS-4 and -5 expressions remained unchanged. TL1A stimulation had no significant effect on ADAMTS-1, -4 and -5 expressions from differentiated THP-1 cells. These observations were also seen in the studies carried out in Chapter 3. When the cells were co-stimulated with TL1A and IFN-γ a reduction in ADAMTS-1 expression was observed but one that was not significantly different to that produced by IFN-γ alone. ADAMTS-4 and -5 expressions were not significantly different after co-stimulation with TL1A and IFN-γ (Figure 4.3).

4.3.1.2 – TL1A and IL-17A

Following on from this, it was of interest to investigate if TL1A could act in synergy with IL-17A to regulate ADAMTS-1, -4 or -5 expressions. To this end, differentiated THP-1 cells were stimulated with either 100ng/ml TL1A or 100ng/ml IL-17A on their own, or co-stimulated with both together. The resulting total RNA was analysed by QPCR to study ADAMTS-1, -4 and -5 gene expressions (Figure 4.4).

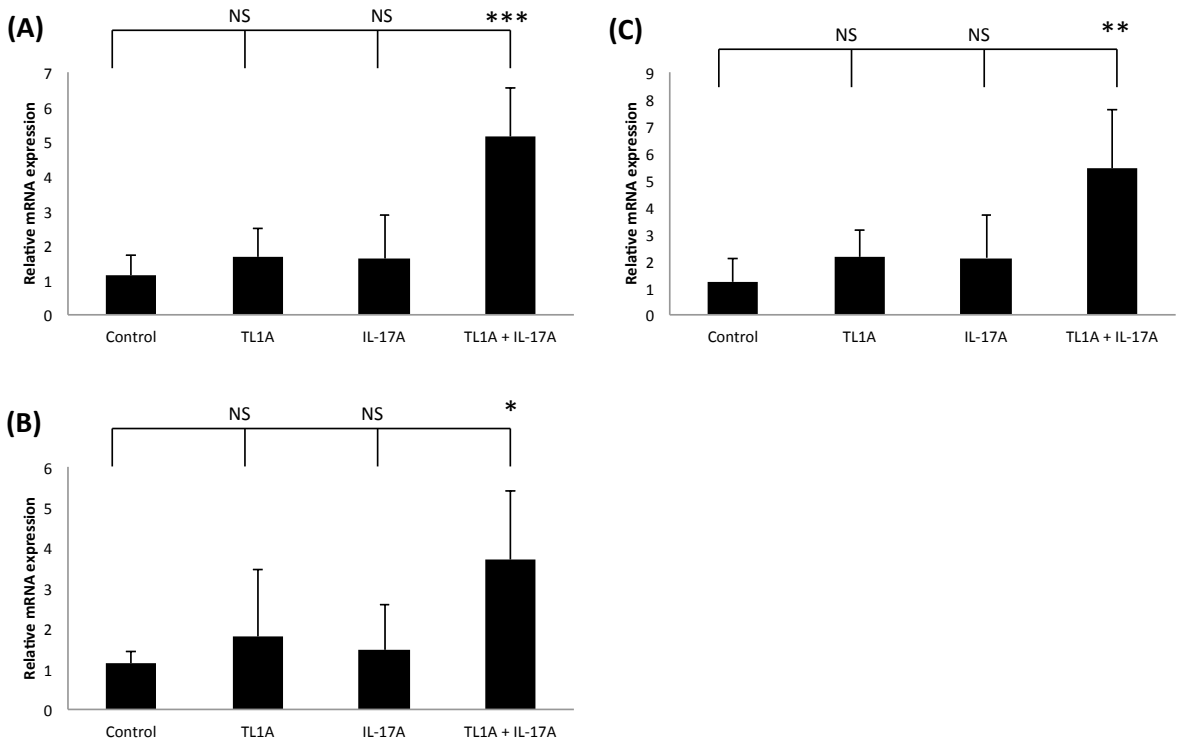


Figure 4.4: TL1A did synergise with IL-17A to regulate the expression of ADAMTS-1, -4 and -5 in differentiated THP-1 cells

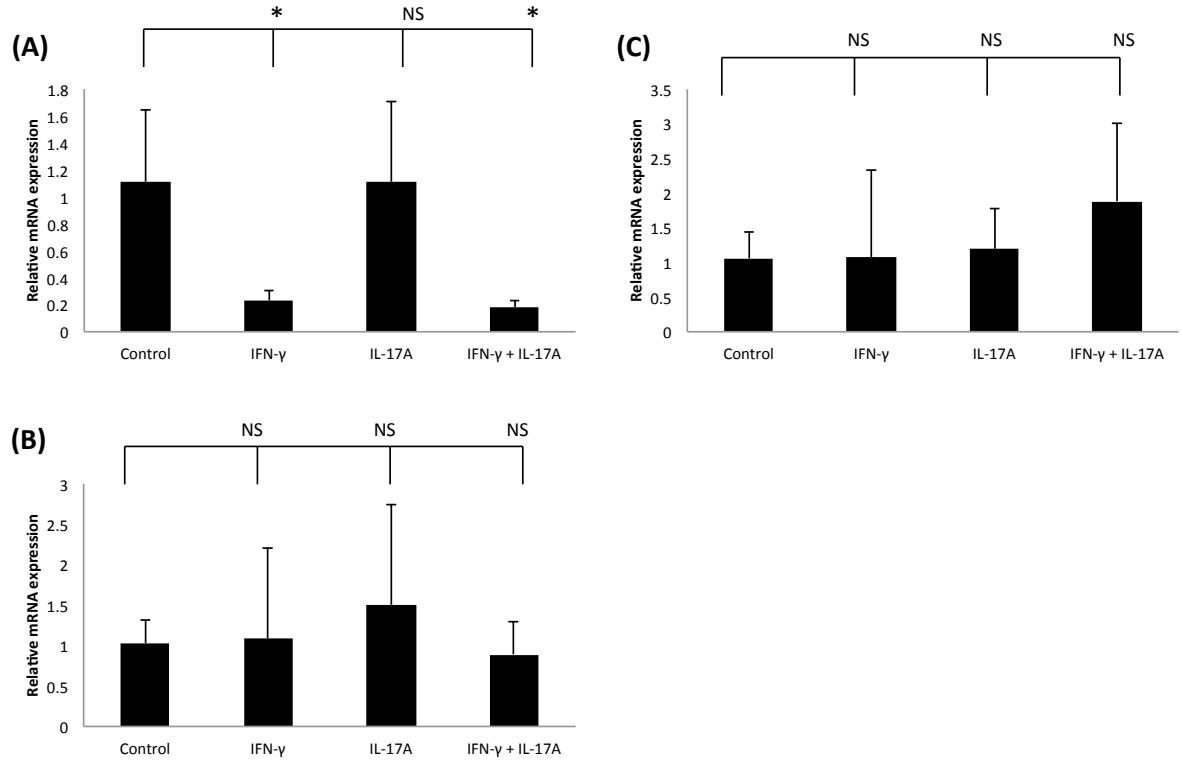
Total RNA from untreated (control) and 24hr cytokine stimulated (100ng/ml TL1A and 100ng/ml IL-17A) differentiated THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. * P= <0.05, **P=<0.01 and ***P=<0.001.

Figure 4.4 shows that when differentiated THP-1 cells were stimulated with TL1A or IL-17A, ADAMTS-1, -4 and -5 expressions remained unchanged. These observations were also seen in the studies carried out in Chapter 3. When the cells were co-stimulated with TL1A and IL-17A a significant increase in ADAMTS-1, -4 and -5 expressions was observed (Figure 4.4).

5.3.1.3 – IL-17A and IFN-γ

Finally, it was interest to investigate if IFN-γ could act in synergy with IL-17A to regulate ADAMTS-1, -4 or -5 expressions. To this end, differentiated THP-1 cells were stimulated

with either 1000U/ml IFN-γ or 100ng/ml IL-17A on their own, or co-stimulated with both together. The resulting total RNA was analysed by QPCR to study ADAMTS-1, -4 and -5 gene expressions (Figure 4.5).



<u>Figure 4.5:</u> IL-17A did not synergise with IFN-γ to regulate the expression of ADAMTS-1, -4 and -5 in differentiated THP-1 cells

Total RNA from untreated (control) and 24hr cytokine stimulated (100ng/ml TL1A and 1000U/ml IFN-γ) differentiated THP-1 cells were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. * P= <0.05.

Figure 4.5 shows that when differentiated THP-1 cells were stimulated with IFN-γ, ADAMTS-1 expression was significantly reduced and ADAMTS-4 and -5 expressions remained unchanged. IL-17A stimulation had no significant effect on ADAMTS-1, -4 and -5 expressions from differentiated THP-1 cells. These observations were also seen in the studies carried out in Chapter 3. When the cells were co-stimulated with IL-17A and IFN-γ a reduction in ADAMTS-1 expression was observed but one that was not significantly

different to that produced by IFN-γ alone. ADAMTS-4 and -5 expressions were not significantly different after co-stimulation with TL1A and IFN-γ (Figure 4.5).

4.3.2 – Cytokine stimulation of HMDMs

After performing the experiments in differentiated THP-1 cells it was shown that TL1A and IL-17A were able to act in synergy to induce the expression of ADAMTS-1, -4 and -5 (Figure 4.5). Following on from these experiments it was of interest to confirm that this response also occurred in primary HMDMs. In order to do this, representative experiments were set up using HMDMs (Figure 4.6).

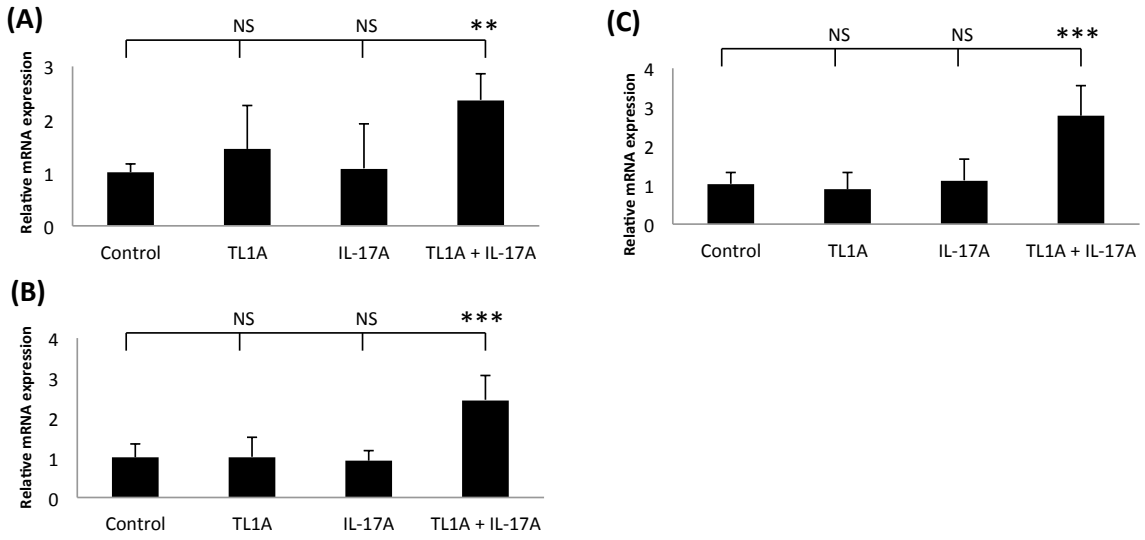


Figure 4.6: TL1A did synergise with IL-17A to regulate the expression of ADAMTS-1, -4 and -5 in HMDMs

Total RNA from untreated (control) and 24hr cytokine stimulated (100ng/ml TL1A and 100ng/ml IL-17A) HMDMs were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using one-way ANOVA. ** P= <0.01 and ***P=<0.001.

Figure 4.6 shows that when HMDMs were stimulated with TL1A or IL-17A, ADAMTS-1, -4 and -5 expressions remained unchanged. When the cells were co-stimulated with TL1A and IL-17A significant increases in ADAMTS-1, -4 and -5 expressions were observed (Figure 4.6).

5.3.4 – Cholesterol uptake assay

The previous experiments that were presented within this chapter have highlighted a synergistic reaction between TL1A and IL-17A during the regulation of ADAMTS-1, -4 and -5 gene expressions in human macrophages (Figures 4.4 and 4.6). TL1A has previously been shown to regulate macrophage uptake of acetylated and oxidised LDL during foam cell formation in atherosclerosis (McLaren et al. 2010a). As the two experiments were both carried out in THP-1 cells it could be possible that the same synergism would extend to regulate other macrophage cellular actions. Macrophage cholesterol uptake assays were utilised to investigate if stimulation with TL1A, IL-17A or TL1A and IL-17A would regulate the amount of acetylated LDL that was taken up by differentiated THP-1 cells (Figure 4.9).

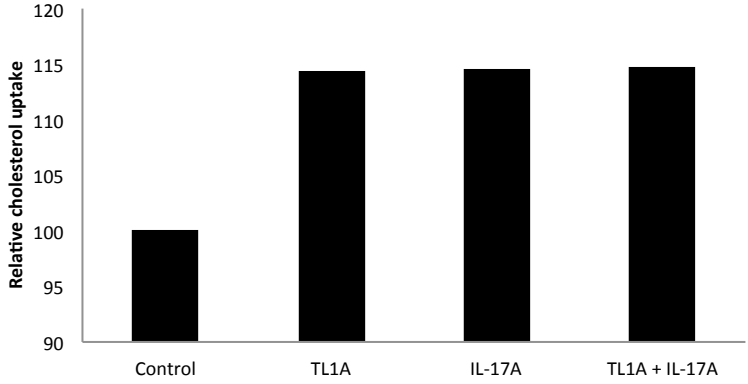


Figure 4.9: TL1A and IL-17A did not synergise to regulate macrophage uptake of Ac-LDL in differentiated THP-1 cells

Differentiated THP-1 cells were incubated with 10µg/ml Dil-AcLDL for 24hrs, either unstimulated (control), or in the presence of 100ng/ml TL1A, 100ng/ml IL-17A or a combination of TL1A and IL-17A. Dil-AcLDL uptake was measured by flow cytometry. The graph displays the average percentage change in Dil-AcLDL uptake in relation to the control (values from untreated samples arbitrarily assigned as 100), from 1 experiment.

Figure 4.9 shows that when differentiated THP-1 cells were stimulated with TL1A the uptake of Dil-acLDL was increased. The percentage uptake was increased by 14.4% by stimulation with TL1A. IL-17A stimulation of differentiated THP-1 cells also showed an increase in the uptake of Dil-acLDL by 14.6%. When the differentiated THP-1 cells were co-stimulated with TL1A and IL-17A together the uptake of Dil-acLDL was also increased in this case. The level of uptake was increased by 14.7%. These data suggest that there was no synergistic reaction between TL1A and IL-17A during the regulation of acLDL uptake in macrophage foam cell formation (Figure 4.9). Unfortunately the experiment was only carried out once; therefore no statistical tests could have been applied to the data.

4.4 – Discussion

Results that have been obtained from previous studies have mainly characterised TL1A and IL-17A as pro-inflammatory agents (Butcher and Galkina 2011; Butcher et al. 2012; Kang et al. 2005; McLaren et al. 2010a). TL1A in combination with IFN-γ, has been shown to increase the expression of MMP-9 in THP-1 cells (Kang et al. 2005). In most studies IL-17A has been suggested to be pro-atherogenic, apart from some exceptions (Butcher et al. 2012; Taleb et al. 2010). In Chapter 3, differentiated THP-1 cells were stimulated with IL-17A and TL1A separately to ascertain if they could regulate gene expression of ADAMTS-1, -4 or -5. Surprisingly, the stimulation with either IL-17A or TL1A did not significantly change the expression of ADAMTS-1, -4 or -5 in macrophages (Chapter 3).

In this experimental chapter the cytokines that have previously been suggested to act in a pro-inflammatory manner were used to co-stimulate differentiated THP-1 cells. TL1A, IL-17A and IFN-γ were added in combinations and the regulation of ADAMTS-1, -4 and -5 expressions was analysed. During this chapter it was shown that when IFN-γ was added in combination with either TL1A or IL-17A no synergistic response was observed in relation to ADAMTS expression (Figures 4.3 and 4.5). However, when TL1A and IL-17A were added together a synergistic response was observed that resulted in the increased expressions of ADAMTS-1, -4 and -5 in differentiated THP-1 cells and HMDMs (Figures 4.4 and 4.6).

This novel finding shows that TL1A and IL-17A acted in synergy to regulate the gene expressions of ADAMTS-1, -4 and -5. This is an important observation because the understanding of how cytokines interact during different disease processes is key in the detailed delineation of the mechanistic actions of inflammatory mediators (Tedgui and Mallat 2006).

The action of the ADAMTS proteases is largely associated with pro-atherogenic endpoints (Salter et al. 2010). In one previous study, ADAMTS-4 and -8 expressions were shown to be up regulated in human atherosclerotic plaques and their expression from differentiated THP-1 cells was increased by stimulation with the pro-inflammatory cytokine, TNF-α (Wagsater et al. 2008). Also in another study, ADAMTS-4 expression was attenuated by stimulation with the anti-atherosgenic cytokine TGF-β (Salter et al. 2011). One other study that looked at the action of ADAMTS-7 during neointima formation and showed that VSMC migration was dependent on the protease (Wang et al. 2009). The results that are presented within this chapter fit with the pro-inflammatory action of the proteases. This is because TL1A and IL-17A are both largely considered pro-inflammatory cytokines that have been shown to exacerbate the disease progression of atherosclerosis (Butcher et al. 2012; Kang et al. 2005).

Recently there is accumulating evidence suggesting that IL-17A and TL1A are involved in the pathogenesis of atherosclerosis via the amplification of inflammation that is induced by other cytokines in synergistic reactions (Ding et al. 2012; Kang et al. 2005). The data obtained within this chapter emphasises the importance of cytokine synergy. Due to the large number of cytokines that are present within the atherosclerotic plaque this could implicate IL-17A and TL1A in a host of synergistic reactions (Tedgui and Mallat 2006). The large number of possible synergisms could go some way to explain why the previous *in vivo* studies on IL-17A have produced some contrasting results. The TL1A and IL-17A synergy to induce ADAMTS expression needs more research and the exact regulatory mechanisms in which IL-17A and TL1A participate need to be further explored.

Firstly, the data presented within this chapter only shows synergistic regulation of ADAMTS-1, -4 and -5 mRNA expressions. It would be of interest to study if TL1A and IL-

17A stimulation could regulate the protein expressions of ADAMTS-1, -4 and -5; this could be carried out by utilising SDS-PAGE and western blot analysis. To take this even further, ADAMTS activity assays could be performed utilising recombinant substrates of ADAMTS proteases. These assays could provide confirmation that the regulation of gene expression by TL1A and IL-17A leads to a physiological increase of ADAMTS activity within the atherosclerotic plaque.

Following on from this, it would be of interest to try and understand the cellular mechanism of how TL1A and IL-17A are able to act in synergy to regulate the expression of ADAMTS-1, -4 and -5. Maybe the stimulation with one cytokine up-regulates the receptor for the other, or maybe one cytokine activates a signalling pathway that the other is dependent on for signal transduction. Possible *in vitro* experiments that could be carried out in differentiated THP-1 cells to aid the understanding of the mechanism are presented below:

- RNA interference (RNAi) targeting NFκB signalling during TL1A and IL-17A stimulation
- RNAi targeting MAPK signalling during TL1A and IL-17A stimulation
- RNAi targeting JAK/STAT signalling during TL1A and IL-17A stimulation
- Co-transfection luciferase assays utilising super-repressor and dominant-negative plasmids for various signalling cascades
- DR3 expression studies following TL1A and IL-17A stimulation
- IL-17 receptor expression studies following TL1A and IL-17A stimulation

Following on from this a standardised series of *in vivo* experiments could be used to try and study these interactions during the disease state. The standardised approach is required as previous studies are not comparable due to the use of different *in vivo* models and different approaches to silencing the effect of IL-17 within these models (von Vietinghoff and Ley 2010). One experiment that could be utilised would be to inject apoE null mice with TL1A and IL-17A, either individually or in combination. The phenotype of the resulting atherosclerotic plaques could be compared to that of control mice. The *in*

vivo model would provide a better understanding of how TL1A and IL-17A regulate disease progression.

The data that was presented in this chapter also looked at the possibility that TL1A and IL-17A synergy could regulate other macrophage cellular actions, such as foam cell formation. A macrophage AcLDL uptake assay was utilised to investigate if TL1A and IL-17A synergy could regulate the macrophage uptake of LDL during atherosclerosis. When the macrophages were stimulated with TL1A the uptake of AcLDL by differentiated THP-1 cells was increased, this was consistent with the previously published literature (McLaren et al. 2010a). The stimulation of differentiated THP-1 cells with IL-17A also caused an increase in AcLDL uptake, this is consistent with the mainly pro-atherogenic role of the cytokine (Butcher et al. 2012). However, when the two cytokines were added together there was a small increase in uptake but no synergistic interaction was observed. The experiment was only carried out once so would need to be repeated to carry out statistical analysis. However, this data suggests that the synergistic response is gene specific to ADAMTS-1, -4 and -5. Further studies of macrophage function are required to see if the synergy can regulate any other genes. One interesting possible experiment would be to carry out a gene array using RNA from macrophages that have been stimulated with TL1A and IL-17A. This would provide a detailed picture of the genes that are activated by the synergistic reaction.

Chapter 5

Signalling pathways underlying the regulation of ADAMTS-1, -4 and -5 expressions by IL-33.

5.1 – Introduction

5.1.1 – IL-33 Signalling

IL-33 has been suggested to signal through the ST2 receptor (Kakkar and Lee 2008). The ST2 receptor was discovered in 1989 and was described as an orphan receptor, as no known ligands had been characterised (Tominaga 1989). The receptor was revealed to participate in many inflammatory processes including stimulating the production of T_h2 cytokines and regulating vascular pathologies (Kakkar and Lee 2008). In 2005, a study revealed that ST2 did have a physiological ligand, IL-33 (Schmitz et al. 2005). IL-33 appears to be a cytokine with a dual function, acting both as a traditional cytokine and as an intracellular nuclear factor, with transcriptional regulatory properties (Kakkar et al. 2012; Miller and Liew 2011). Macrophages constitutively express the ST2 receptor and IL-33 activation of ST2 receptors on macrophages can amplify an IL-13 driven polarization of macrophages towards a M2 phenotype producing Th2 cytokines (Miller and Liew 2011). The activation of the ST2 receptor by IL-33 has also been shown to activate multiple signalling pathways in various cell types (reviewed in Table 5.1).

Table 5.1: Studies that have investigated the mechanism of IL-33 signal transduction

Reference	Findings	Methods	Cell Type
(Brint *et al.* 2002)	Activated the activator protein (AP-1), c-Jun, JNK, ERK, p38.	Phosphorylation western blots; luciferase p38 MAPK activation assay; increase in IL-4 expression blocked by JNK inhibitor (SP600125).	Murine thymoma EL4; mouse masT-cell line (P815); naive T-cells; HEK293 cells.
(Funakoshi-Tago *et al.* 2008)	Activated p38, JNK, NFκB in TRAF6 dependent mechanism; ERK activation was TRAF6 independent.	Mouse TRAF6$^{-/-}$ MEK cells; phosphorylation western blots; luciferase NFκB activation assays.	Murine Embryonic Fibroblasts (WT and TRAF6$^{-/-}$).
(Yagami *et al.* 2010)	ERK and p38 activated in endothelial cells; ERK only in epithelial cells; IL-8 regulation was p38 dependent in endothelial cells and ERK dependent in epithelial cells.	Phosphorylation western blots; pharamacological inhibitors.	Normal human bronchial epithelial cells and human microvascular endothelial cells.
(Tare *et al.* 2010)	Activated NFκB, JNK, p38 but not ERK; IL-13 regulation was dependent on NFκB but not p38 or JNK.	Bio-plex™ multiplex phosphorylation detection system; pharmacological Inhibitors.	Basophil-like chronic myelogenous leukemia cell line (KU812).
(Kamekura *et*	IL-8 regulation was	Corticosteroid	Nasal epithelia.

al. 2012)	dependent on ERK, p38, JNK, NFκB and epidermal growth factor receptor.	treatment; pharmacological inhibitors.	
(Funakoshi-Tago *et al.* 2011)	IL-33 activated JAK2; A JAK2 inhibitor reduced NFκB activation but not ERK, JNK and p38 activation.	Phosphorylation western blots; pharmacological inhibitors.	Murine peritoneal Macrophages.
(Choi *et al.* 2009)	IL-33 activated Akt and eNOS; IL-33 induced angiogenesis and vascular hyperpermeability was dependent on PI3K/Akt/eNOS.	Phosphorylation western blots; pharmacological inhibitors.	Human umbilical vein endothelial cells.

5.1.2 – Introduction to the signalling pathways activated by IL-33

5.1.2.1 - MAPK Signalling

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that are implicated in the regulation of various cellular activities including cell proliferation, differentiation, survival, death and transformation (Kim and Choi 2010). An overview of the MAPK signalling cascade is presented in Figure 5.1. The mammalian MAPK family is made up of three main distinct pathways that comprise at least three common components; a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK (Dhillon et al. 2007; Kim and Choi 2010). The pathways act as a cascade of sequential phosphorylation causing activation of downstream mediators. The major distinct pathways are termed the extracellular signal-regulated kinases (ERK), p38 MAPKs and c-Jun NH_2-terminal kinases (JNK) (Dhillon et al. 2007). Each of the specific components within the pathways has several different isoforms they are termed ERK-1-8, p38-α, -β, -γ, -δ and JNK-1-3 (Dhillon et al. 2007; Schaeffer and Weber 1999). The MAPK pathways are

activated by external factors causing a series of binary interactions between specific kinase components and scaffolding proteins within the cell membrane (Kim and Choi 2010). These activations then lead to intracellular cascades that result in gene regulation and cellular changes via specific transcription factors such as ETS-like transcription factor-1 (Elk-1), c-Jun, activating transcription factor-2 (ATF2) and p53 (Kim and Choi 2010).

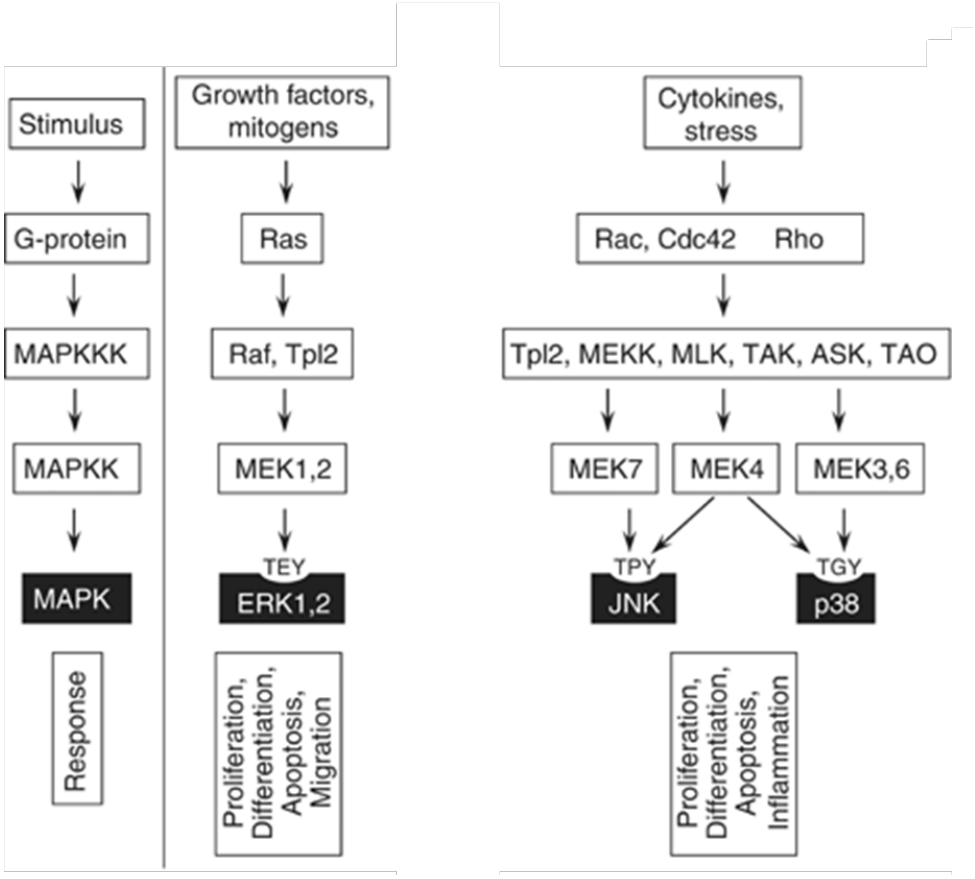


Figure 5.1: An overview of the MAPK signalling cascade (Dhillon et al. 2007)

MAPK pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis. MAPK pathways are comprised of a three-tier kinase module in which a MAPK is activated upon phosphorylation by a MAPKK, which in turn is activated when phosphorylated by a MAPKKK. The three major MAPK pathways are termed ERK, p38 and JNK.

5.1.2.2 - PI3K/Akt Signalling

The phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathway regulates many cellular functions including proliferation, growth, survival and mobility (Morgensztern and McLeod 2005). An overview of the PI3K signalling cascade is presented in Figure 5.2. The signalling through the pathway begins when tyrosine kinase receptors are activated on the extracellular surface of the cell membrane by growth factors or cytokines. The activation of tyrosine kinase receptors involves autophosphorylation of tyrosine residues and transphosphorylation of adaptor proteins (Morgensztern and McLeod 2005). Once activated the tyrosine residues or adaptor proteins can phosphorylate PI3K, bringing the enzyme to the inner surface of the membrane and causing the p110 catalytic unit to become active (Morgensztern and McLeod 2005). PI3K acts as lipid kinase and is able to convert phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3). Subsequent signalling downstream from these events involves serine/theronine kinases such as RAC-alpha serine/threonine-protein kinase (Akt) and phosphoinositide-dependent kinase-1 (PDK1) (Manning and Cantley 2007; Morgensztern and McLeod 2005). When Akt1 binds to PIP_3 a conformational change occurs that allows PDK1 to phosphorylate Akt1 at Thr308, this along with a further phosphorylation, carried out by an unknown kinase at Ser473, results in full Akt1 activation (Manning and Cantley 2007). Akt is able to regulate many target proteins in the control of apoptosis, migration and cell proliferation (Manning and Cantley 2007).

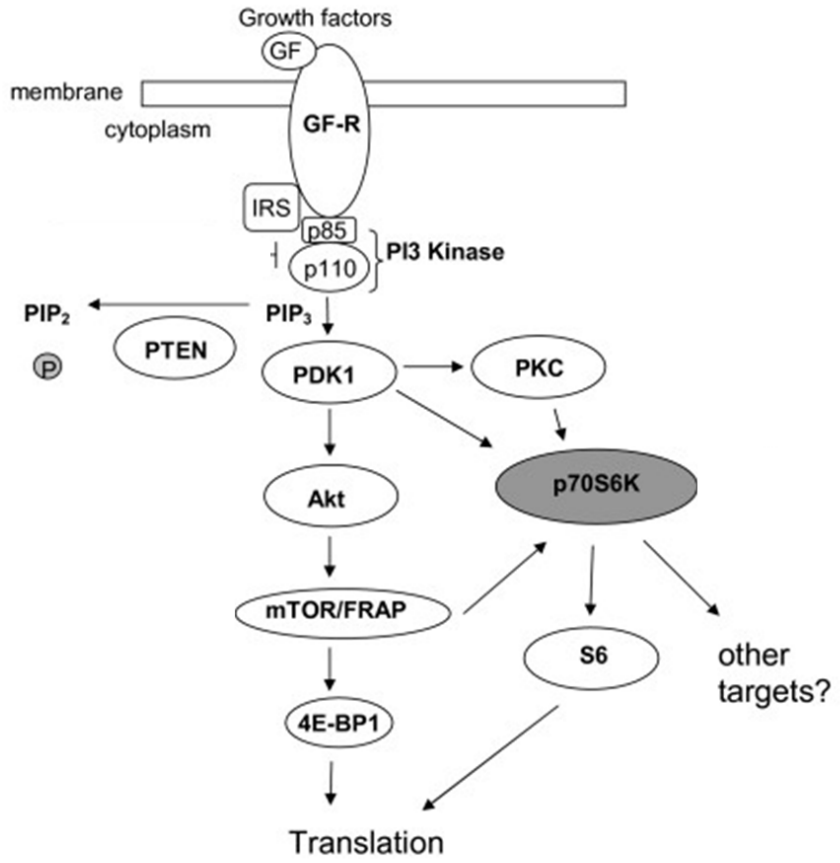


Figure 5.2: An overview of the PI3K signalling cascade (Heinonen *et al.* 2008)

PI3K acts as lipid kinase and is able to convert PIP_2 to PIP_3. Subsequent signalling downstream from these events involves serine/theronine kinases such as Akt, PKC and PDK1. The PI3K signalling cascade is able to regulate many target proteins in the control of apoptosis, migration and cell proliferation.

5.1.2.3 - NFκB Signalling

NFκB proteins consist of a family of structurally-related transcription factors that include p50, p65, c-Rel, p52 and RelB, all of which have a conserved N-terminal Rel homology domain (RHD) that contains DNA binding regions (Nogueira et al. 2011). Of the family, only p65, RelB and c-Rel can act as transcriptional activators solely, others usually function as dimers (Pamukcu et al. 2011). NFκB is usually found in the cytosol in an inactive state complexed with an inhibitory protein called IκB (Nogueira et al. 2011). Upon activation of the pathway IKKβ is able to phosphorylate IκB, causing it to dissociate from the protein complex (Nogueira et al. 2011). The active NFκB dimer is usually made

up of mainly p50, p65 or c-Rel can now translocate to the nucleus and act on a large range of target genes (Nogueira et al. 2011). The NFκB pathway has been implicated in cell survival/apoptosis, cell growth and inflammation (Pamukcu et al. 2011). NFκB activation is outlined in Figure 5.3.

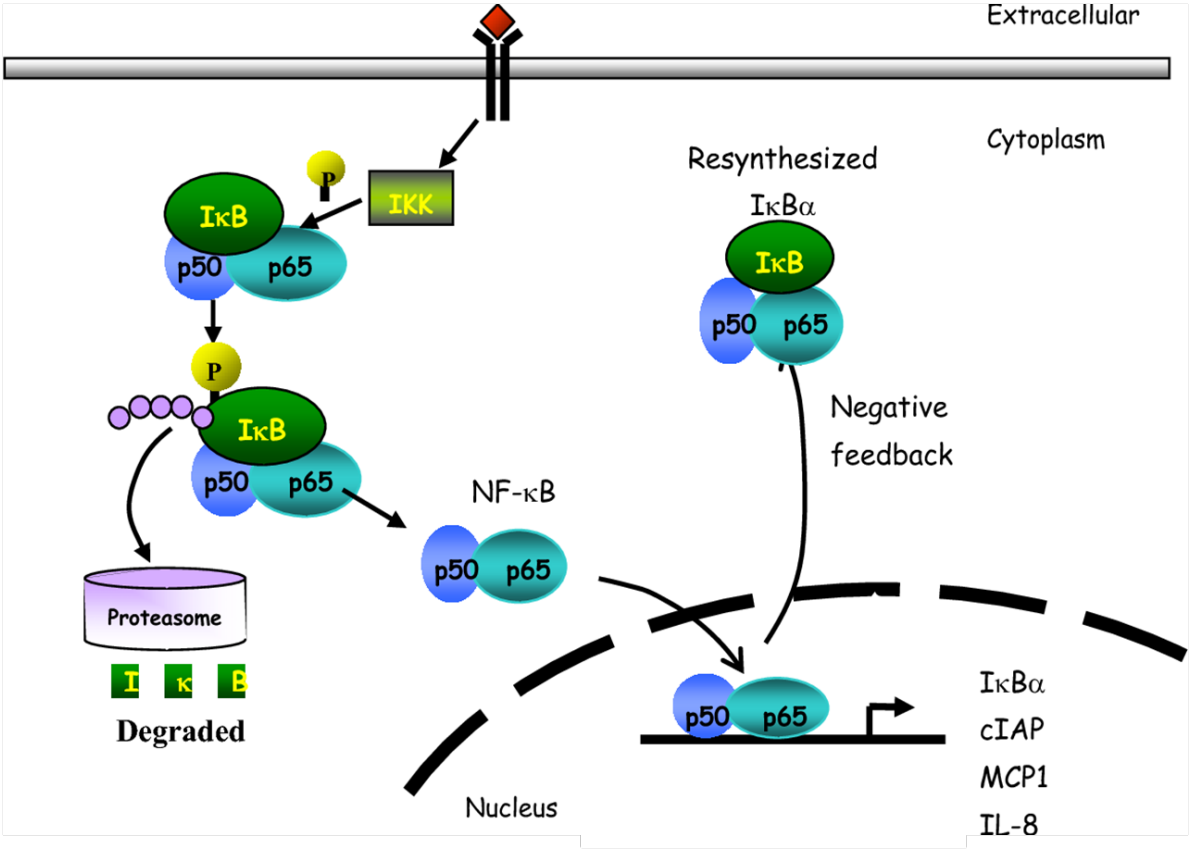


Figure 5.3: An overview of NFκB activation (Pamukcu et al. 2011)

In an un-activated state NFκB is usually complexed with an inhibitory protein called IκB. Upon activation of the pathway IKK is able to phosphorylate IκB, causing it to dissociate from the protein complex. The active NFκB dimer can now translocate to the nucleus and act on a large range of target genes. The NFκB pathway has been implicated in cell survival/apoptosis, cell growth and inflammation.

5.1.2.4 - JAK/STAT Signalling

The JAK-STAT pathway has been shown to be conserved within all vertebrates and is even found in some metazoas, the pathway is implicated in the regulation of survival, proliferation, and differentiation of various cell and tissue types (Schindler et al. 2007; Vera et al. 2011). In mammals, four different isoforms of JAK (JAK1-3 and Tyk2) and seven

isoforms of STAT (STAT1-4, 5A, 5B and 6) have been characterised (Schindler et al. 2007; Vera et al. 2011). An overview of the JAK/STAT signalling cascade was presented in Figure 1.8 (General Introduction). The signalling cascade begins with the extracellular binding of a cytokine to its receptor complex causing dimerisation, the formation of the receptor subunit dimer causes activation of JAKs by tyrosine phosphorylation (Vera et al. 2011). The active JAKs are able to phosphorylate tyrosine residues of the cytoplasmic receptor domain causing binding sites for STAT proteins to be revealed (Vera et al. 2011). Once bound JAK-mediated phosphorylation of a crucial tyrosine reside near the C-terminus causes STAT1 to dissociate from the receptor and translocate to the nucleus and act on specific targets to modulate gene expression (Vera et al. 2011). In addition, phosphorylation of STAT-1 at serine 727 is required for maximal activity of gene transcription (McLaren and Ramji 2009).

5.1.3 – Cytokine regulation of ADAMTS expression and signalling cascades

As previously reviewed in the General Introduction, Chapter 3 and Chapter 4, cytokine stimulation has been shown to regulate ADAMTS expression in many different cell types. Some previous studies have investigated which signalling cascades are involved in transducing a cytokine response through to ADAMTS gene regulation. A study carried out within our laboratory showed that TGF-β stimulation of macrophages inhibited ADAMTS-4 expression and promoter activity (Salter et al. 2011). It was elucidated that TGF-β regulation of ADAMTS-4 required Smads, p38-MAPK and c-Jun (Salter et al. 2011). In a recent co-culture study, a combination of osteoarthritis patient's subchondral bone osteoclasts and normal articular cartilage chondrocytes resulted in significantly increased expression of ADAMTS-4 and ADAMTS-5 (Prasadam et al. 2012). Upregulation of ADAMTS under these conditions was correlated with activation of the ERK1/2 signalling pathway as addition of the ERK inhibitor PD98059 reversed the over-expression of ADAMTS in the co-cultures (Prasadam et al. 2012). Another recent study showed that in fibroblast-like synoviocytes expression of ADAMTS-4 was increased by addition of IL-6. The increased expression was inhibited by the addition of a MEK-1/2 inhibitor (Mimata et al. 2012).

5.1.4 – RNA Interference (RNAi)

RNAi is a process by which the mRNA expression of a gene can be specifically and selectively reduced (Figure 5.4). The technique is commonly used during *in vitro* and *in vivo* experiments to study the role of specific genes during cellular processes (Meister and Tuschl 2004). The method of mediating the RNAi effect involves introducing double stranded (ds) RNA molecules into the cell which are then processed to create a specific RNA-interfering silencing complex (RISC) (Rao et al. 2009b). There are two methods that have been utilised for the cellular delivery of dsRNA molecules:

Firstly, a transient transfection of siRNA molecules is carried out using cationic transfection reagents (Rao et al. 2009b). For successful transfection, a negatively charged nucleic acid must come into contact with a cell membrane that also carries a net negative charge (Dalby et al. 2004). Cationic liposome formulations function by forming complexes with nucleic acid molecules, allowing them to overcome the electrostatic repulsion of the cell membrane and to be taken up by the cell (Dalby et al. 2004).

Secondly, shRNAs are delivered into the cells by utilising plasmid vectors that are directed by RNA polymerase III to provide a stable expression of shRNA by the hosts cellular machinery (Shi 2003). The use of plasmid vectors to deliver shRNA into cells will be expanded in Chapter 6.

Once the dsRNA is present within the cell it undergoes a series of processing steps that ultimately lead to specific mRNA cleavage (Meister and Tuschl 2004). Firstly, the dsRNA or shRNA interacts with the RNase III endonuclease Dicer. The action of Dicer produces two strands of complimentary siRNA around 21-23 nucleotides in length (Meister and Tuschl 2004). The siRNA then associates with RISC which allows the two strands of siRNA to separate. The RISC-siRNA complex is now active and can execute specific mRNA cleavage of the target (Rao et al. 2009b; Rutz and Scheffold 2004; Shi 2003).

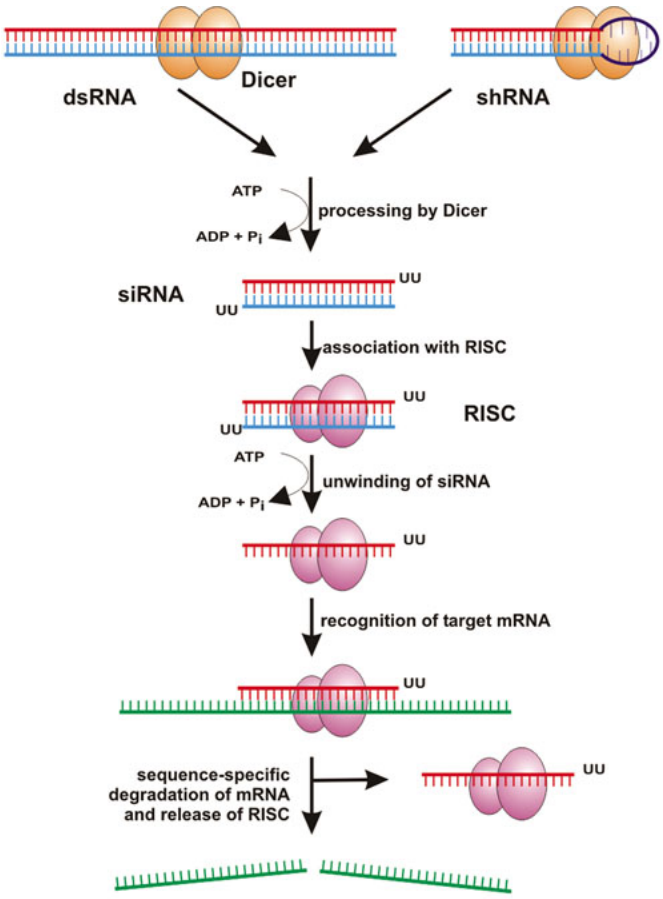


Figure 5.4: **RNA interference mechanism (Rutz and Scheffold 2004)**

Long dsRNA or shRNA is processed by Dicer to form siRNA, which associates with RISC and mediates target sequence specificity for subsequent mRNA cleavage.

5.2 – Experimental Aims

The primary aims of this chapter were to ascertain if the regulation of ADAMTS-1, -4 and -5 by IL-33 was occurring mainly at a gene transcriptional level. Following on from this, it was of interest to delineate the intracellular signalling cascades that are involved in the transduction of the response. An overview of the experimental strategy is displayed in Figures 5.5 – 5.7.

Firstly, it was investigated if IL-33 was producing its regulation over ADAMTS-4 expression by causing a reduction in promoter activity (only ADAMTS-4 promoter construct was available for these studies). ADAMTS-4 luciferase gene reporter assays were utilised to

study the promoter activity after IL-33 stimulation. THP-1 cells are notoriously difficult to transfect with recombinant plasmids, therefore necessitating the use of another cell line for these investigations. RAW264.7 cells are a mouse macrophage cell line, as these cells were available within the laboratory and are easy to transfect, they were chosen for any DNA/plasmid based transfection studies performed within this chapter.

Secondly, the involvement of the ST2 receptor during the response was analysed by stimulating mouse $ST2^{-/-}$ BMDMs with IL-33. The IL-33 stimulation was also carried out on WT BMDMs; the regulation of ADAMTS-1, -4 and -5 was then compared between the two groups.

Following on from these experiments, it was of interest to elucidate which signalling cascades were activated by IL-33 stimulation of macrophages, as this will allow a greater understanding of the intracellular mechanism behind the reduction of ADAMTS-1, -4 and -5 expressions. Phosphorylation of specific signalling molecules were analysed using SDS-PAGE and western blot after IL-33 stimulation of THP-1 cells. Also, co-transfection luciferase gene reporter assays were used to ascertain the activation state of NFκB after mIL-33 stimulation of RAW264.7 cells. Co-transfection studies were also used to investigate if the NFκB pathway was involved in transducing IL-33s regulation over ADAMTS-4 expression.

In the light of the modest changes observed from the transfection studies and the lack of a luciferase reporter construct for ADAMTS-1 or -5, it was decided that RNAi should be employed to target specific components of various signalling cascades to try and delineate the response. After reviewing the available literature, some cellular targets were outlined for the mechanistic studies presented within this chapter (Table 5.2). The cellular targets were chosen to try and investigate, for the first time, which signalling pathways were responsible for the transduction of IL-33s regulation over ADAMTS-1, -4 and -5. Key components from each chosen pathway were targeted using RNAi to ascertain if the reduction in ADAMTS-1, -4 and -5 expressions by IL-33 were dependent on that specific cascade. After performing RNAi, IL-33 stimulation was carried out and the

expression of ADAMTS-1, -4 and -5 was studied by QPCR in comparison to the suitable controls.

Table 5.2: **Cellular targets chosen for the mechanistic studies into the underlying signalling pathways of IL-33 regulation over ADAMTS-1, -4 and -5 expressions.**

Gene	RNAi method
ERK-1	shRNA
ERK-2	shRNA and siRNA
p38	shRNA
JNK-1/2	siRNA
c-Jun	siRNA
PI3K-γ	shRNA
PI3K-δ	shRNA
p50 and p65	siRNA
JAK1 and 2	siRNA

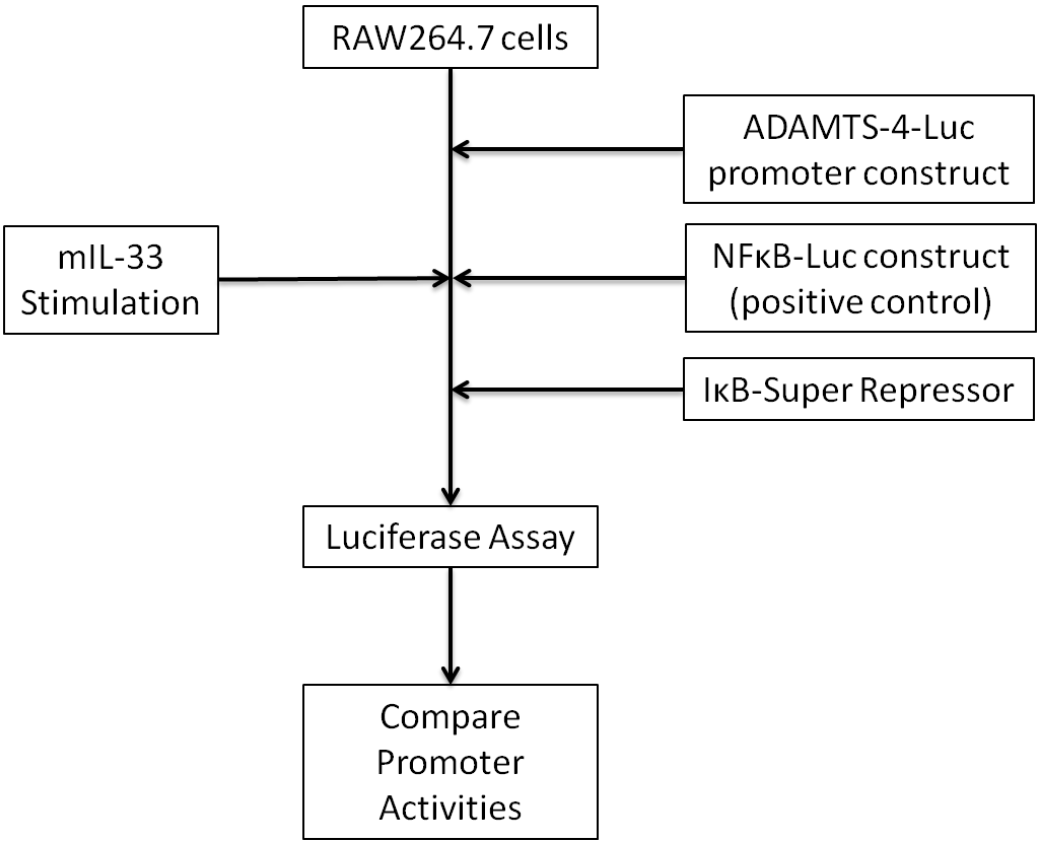


Figure 5.5: **Transfection based studies experimental strategy**

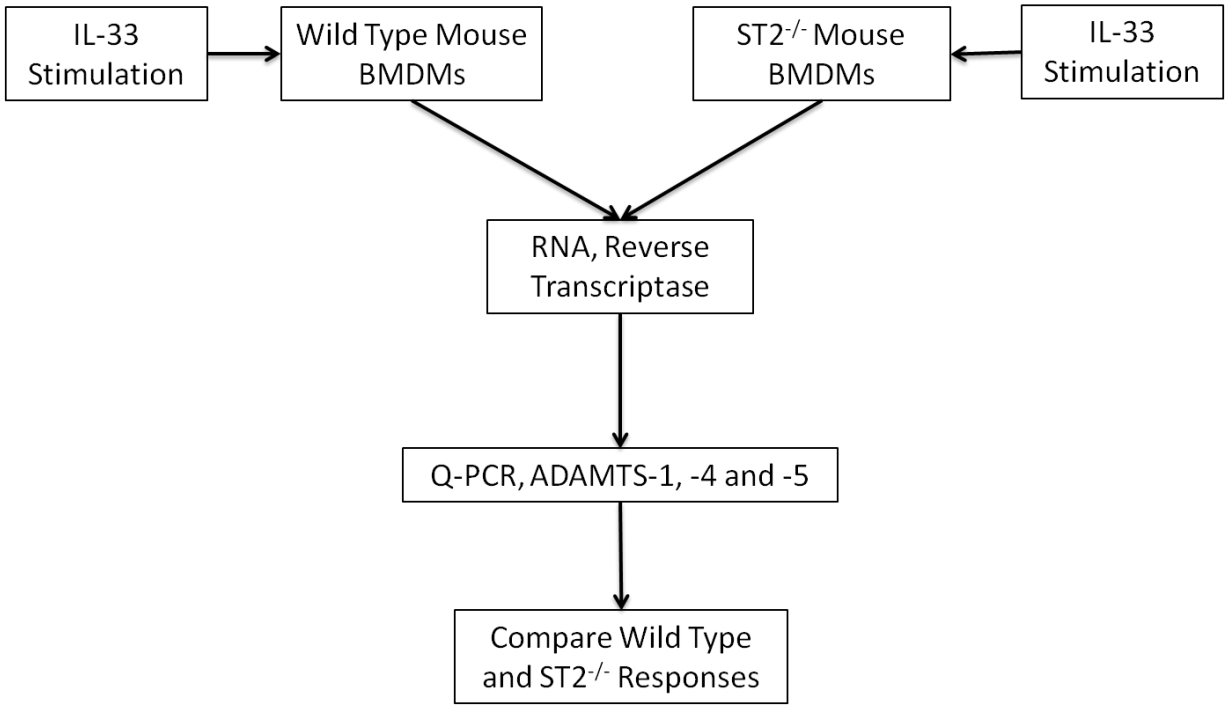


Figure 5.6: **ST2$^{-/-}$ mouse experimental strategy**

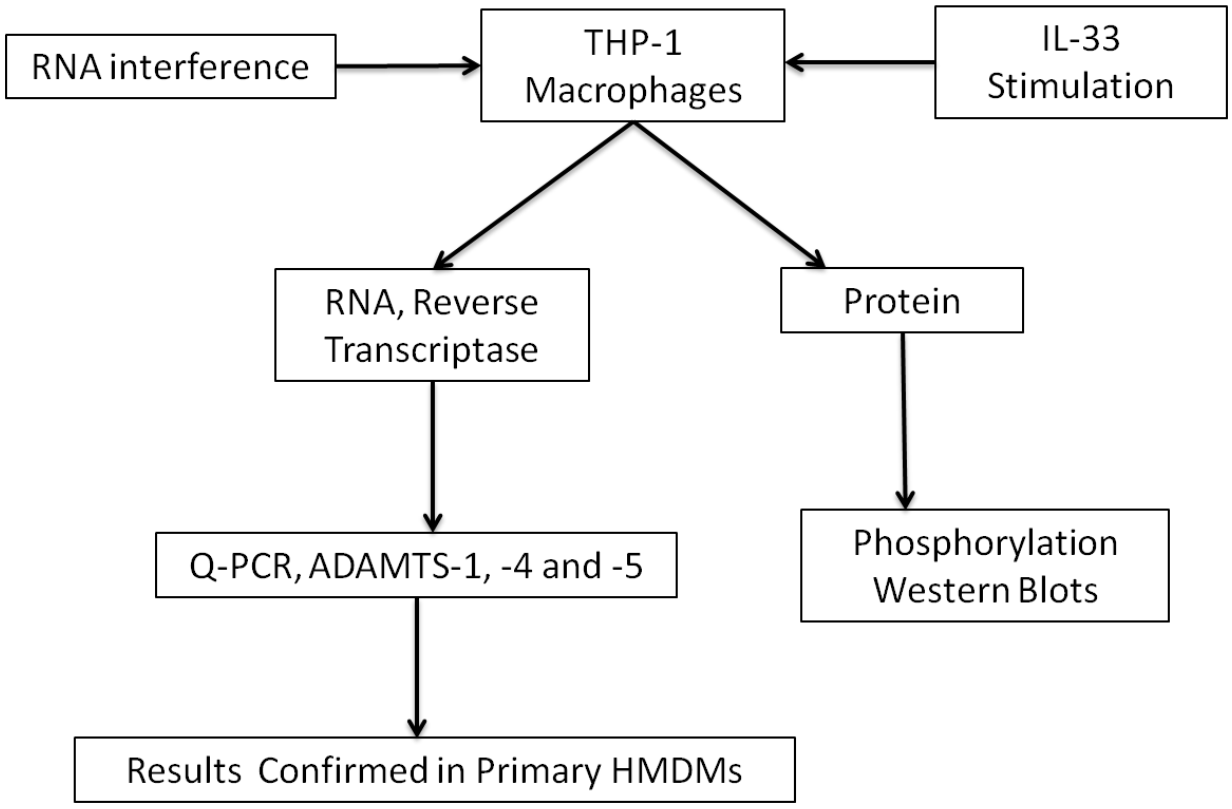


Figure 5.7: **THP-1 cell experimental strategy**

5.3 – Results

5.3.1 – ADAMTS-4 luciferase gene reporter assay

In order to try and investigate if IL-33 was causing regulation via gene transcription, a human ADAMTS-4 promoter construct was used (Mizui et al. 2000). The promoter construct was called p-789-Luc and spanned the region -383/+406 of the ADAMTS-4 promoter (Figure 5.8).

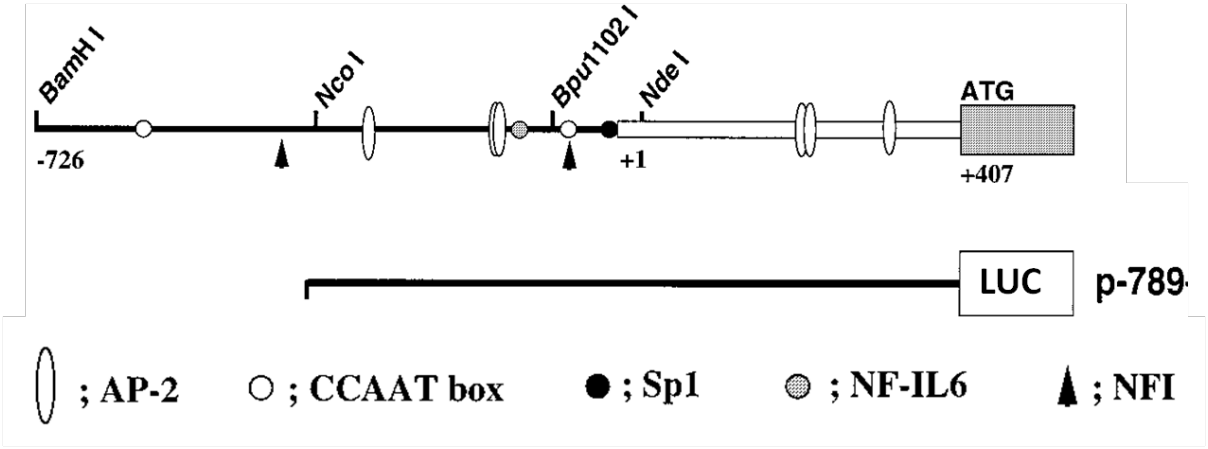


Figure 5.8: Schematic of the p-789-Luc promoter construct (Mizui et al. 2000)

The p-789-Luc, ADAMTS-4 promoter construct. The Numbers indicate the relative positions with respect to the tentative transcription start point. Some transcription factor binding sites are detailed including AP2, CCAAT box, Sp1 and NF-IL6.

Before the action of IL-33 over the ADAMTS-4 promoter activity could be investigated, it was necessary to determine if the cytokine response was conserved between THP-1 and RAW264.7 cells. As previously mentioned RAW264.7 cells were used for transfection-based studies because THP-1s cannot be transfected with exogenous DNA at high efficiency. RAW264.7 cells were stimulated with 10ng/ml mIL-33 for 24hrs and the expression of mADAMTS-4 mRNA was analysed using Q-PCR (Figure 5.9). The 24hr time-point was chosen to mimic the experiments carried out within Chapter 3. As expected, mIL-33 stimulation down-regulated the expression of mADAMTS-4 mRNA in RAW264.7 cells, verifying the use of this cell line in our investigations.

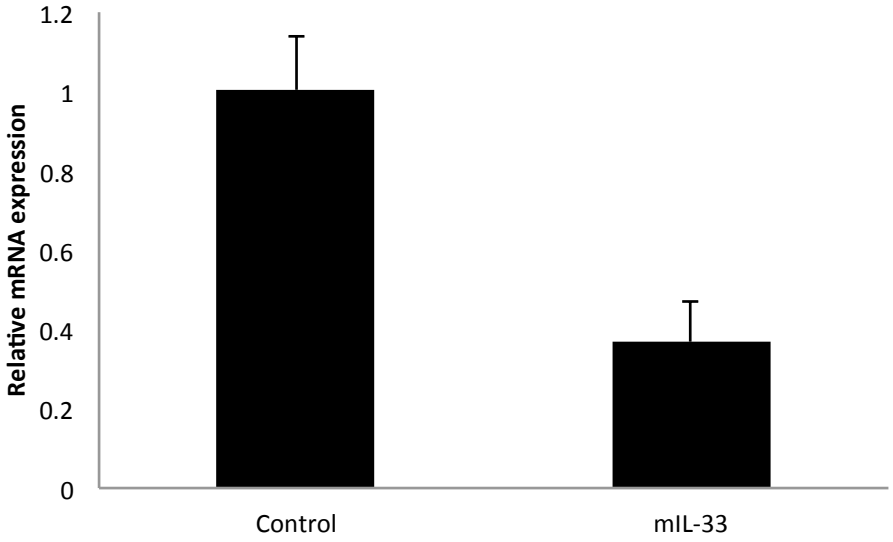


Figure 5.9: mIL-33 stimulation reduced the expression of mADAMTS-4 in RAW264.7 cells

RAW264.7 cells were either left untreated or stimulated with 10ng/ml mIL-33 for 24hrs. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis for mADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (values from untreated samples arbitrarily assigned as 1), from 1 experiment.

Following on from this initial experiment, ADAMTS-4 promoter activity was analysed after mIL-33 stimulation of RAW264.7 cells (Figure 5.10).

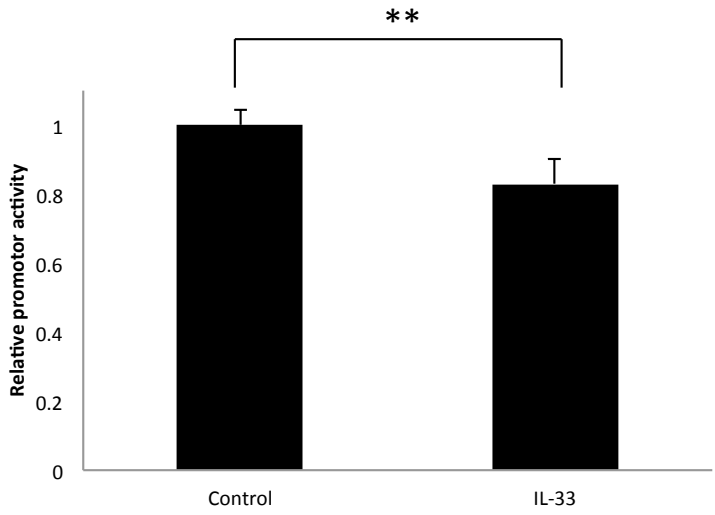


Figure 5.10: mIL-33 stimulation of RAW264.7 cells caused a reduction in ADAMTS-4 promoter activity

RAW264.7 cells were transfected with 2µg of the ADAMTS-4 promoter (p-789-Luc) using Superfect™. Cells were either left untreated or stimulated with mIL-33 for 24hrs. Cells were harvested using passive lysis buffer and assayed for luciferase activity. Relative luciferase activity (RLA) readings were normalised to protein concentration as determined by the BCA protein assay (values from untreated samples arbitrarily assigned as 1). The graph displays data from three independent experiments. Statistical analysis was carried out using the Students t-test. **P=<0.01.

Figure 5.10 showed a significant yet small reduction in ADAMTS-4 promoter activity after RAW264.7 cells were stimulated with 10ng/ml mIL-33 for 24hrs. These data suggest that one mechanism by which IL-33 regulates the expression of ADAMTS-4 is via gene transcription; another possible mechanism is modulation of mRNA stability and miRNA expression.

5.3.2 – IL-33 stimulation of mouse ST2$^{-/-}$ BMDMs

In order to study if the ST2 receptor was having a role during IL-33's regulation of ADAMTS-1, -4 and -5, mouse ST2$^{-/-}$ BMDMs were stimulated with mIL-33 and the expressions of mADAMTS-1, -4 and -5 were analysed in comparison to WT controls. The mouse bones were kindly donated by Prof. E Liew and the BMDMs were cultured and

prepared during work for a previously published study (McLaren et al. 2010c). Unfortunately, due to the lack of availability of mouse BMDMs, only enough cells were obtained to allow the investigation of ADAMTS-1 and -4 expressions from the BMDMs. Also it was only possible to repeat the experiment twice; this unfortunately does not allow the use of statistical analysis on the data. The results obtained during these experiments are presented in Figure 5.11.

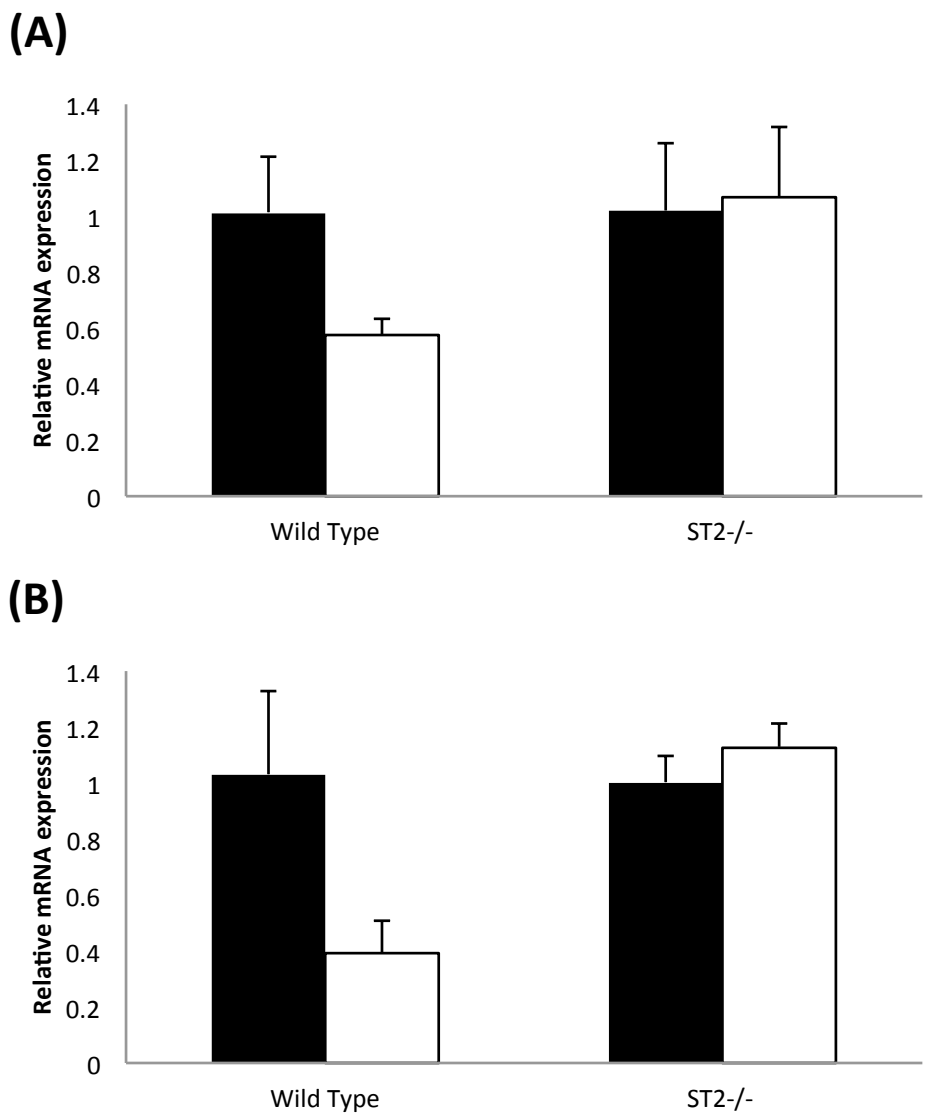


Figure 5.11: IL-33 inhibited the expression of mADAMTS-1 and -4 via the ST2 receptor
Mouse wild type and ST2$^{-/-}$ BMDMs were either left untreated (filled bars) or stimulated with 10ng/ml mIL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = mADAMTS-1 and B = mADAMTS-4. The graphs display the average fold change in mRNA expression in relation

to the control (values from untreated samples arbitrarily assigned as 1), from 2 independent experimental repeats.

From Figure 5.11, it was observed that mIL-33 exhibited the same pattern of regulation over mADAMTS-1 and -4 that was previously shown in human THP-1 cells and HMDMs (Chapter 3). Thus, after 24hrs of mIL-33 stimulation (10ng/ml) the expression of mADAMTS-1 and -4 was reduced within WT BMDMs (Figure 5.11, panels A and B). However, when the 24hr mIL-33 stimulation (10ng/ml) was extended to the $ST2^{-/-}$ BMDMs, no change was observed to mADAMTS-1 and -4 expressions (Figure 5.11, panels A and B), thereby indicating a requirement for the ST2 receptor in the response.

5.3.3 – Determining the activation state of key components from various signalling cascades after IL-33 stimulation of macrophages

5.3.3.1 – ERK

In order to determine the phosphorylation state of ERK after IL-33 stimulation, an antibody was used that detects phosphorylation of p44/42 MAPK at Thr202/Tyr204. Differentiated THP-1 cells were stimulated for 24hrs with 10ng/ml IL-33. The 24hr duration of stimulation was chosen on the basis of the response presented in Chapter 3. The resulting western blot is presented in Figure 5.12.

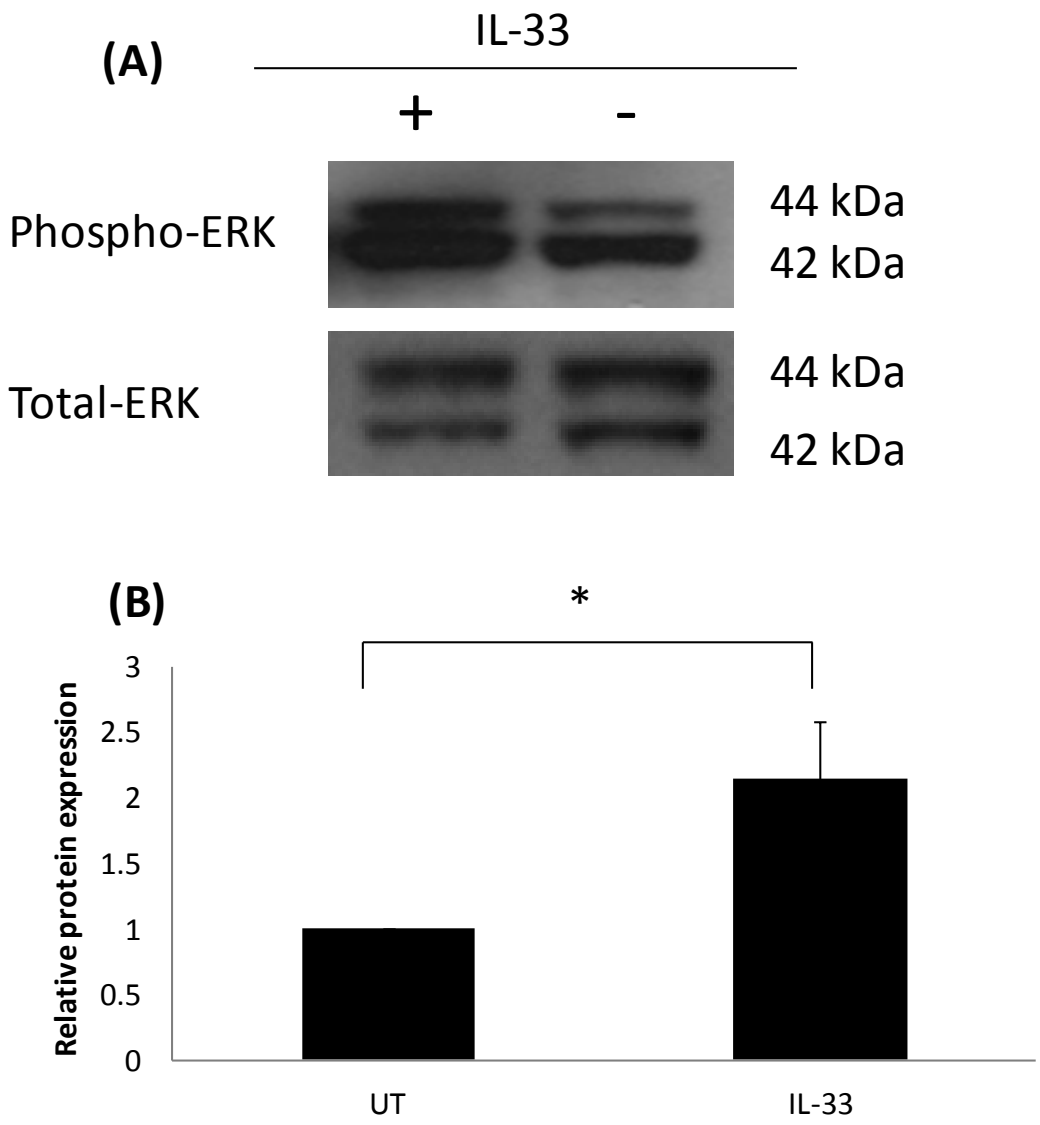


Figure 5.12: ERK-1 and -2 were activated in response to IL-33 stimulation of differentiated THP-1 cells

Differentiated THP-1 cells were left untreated (control) or stimulated with 10ng/ml IL-33 (24hr). Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against phosphorylated-ERK and total-ERK. Panel A = Representative blot and Panel B = Densitometry graph. The image presented is representative of the 3 experimental repeats. The graph displays the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P= <0.05.

Figure 5.11 shows that 24hrs of IL-33 stimulation (10ng/ml) produced a significantly increased level of phosphorylation of ERK-1/2 compared to the control (Figure 5.12). The expression levels of total ERK-1 and -2 in differentiated THP-1 cells were unchanged after IL-33 stimulation.

5.3.3.2 – p38α MAPK

Following on from investigating ERK activation, it was of interest to study if another MAPK pathway, p38, was also activated by IL-33 stimulation of THP-1 cells. An antibody was utilised that detects phosphorylation of p38α MAPK at Thr180/Tyr182. Differentiated THP-1 cells were stimulated for 24hrs with 10ng/ml IL-33. Again, the 24hr duration of stimulation was chosen on the basis of the response presented in Chapter 3. The resulting western blot is presented in Figure 5.13.

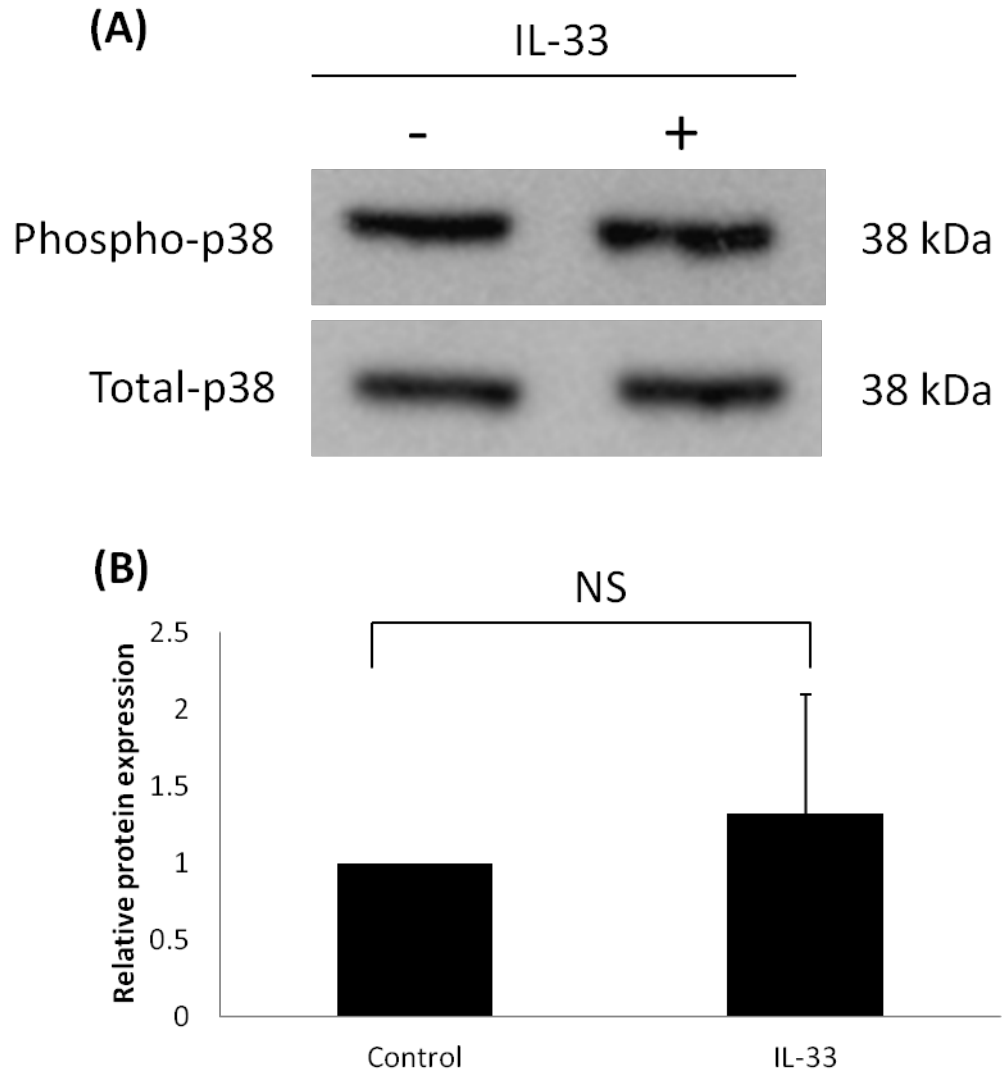


Figure 5.13: p38α was not activated in response to IL-33 stimulation of differentiated THP-1 cells

Differentiated THP-1 cells were left untreated (control) or stimulated with 10ng/ml IL-33 (24hr). Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against phosphorylated-p38α and total-p38α. Panel A = Representative blot and Panel B = Densitometry graph. The graph displays the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 5 independent experimental repeats. The blot presented is representative of the 5 experimental repeats. Statistical analysis was performed using the Student's t-test.

Figure 5.13 shows that 24hrs of IL-33 stimulation (10ng/ml) produced no change to the level of phosphorylation to p38α compared to the control, suggesting that p38α was not

activated. The expression level of total p38α in differentiated THP-1 cells was unchanged after IL-33 stimulation (Figure 5.13).

5.3.3.3 – c-Jun

c-Jun is a transcription factor that is a downstream target of JNK within the MAPK signalling cascade (Kim and Choi 2010). Initially the phosphorylation state of JNK was also planned to be investigated. However, subsequent problems with the immuno-detection of phosphorylated-JNK meant that activation of a downstream target (c-Jun) was utilised. In order to determine the phosphorylation state of c-Jun after IL-33 stimulation, an antibody was used that detects phosphorylation of the protein at Ser63. Differentiated THP-1 cells were stimulated for 24hrs with 10ng/ml IL-33. The resulting western blot is presented in Figure 5.14.

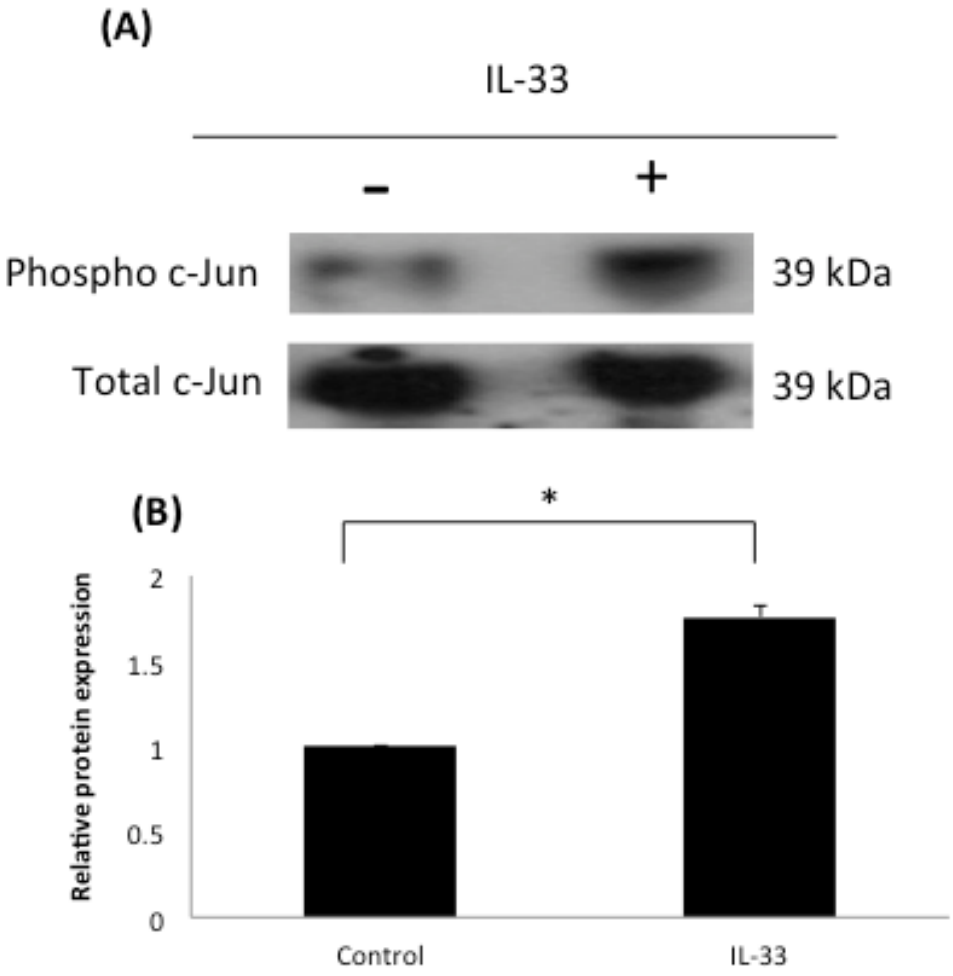


Figure 5.14: c-Jun was activated in response to IL-33 stimulation of differentiated THP-1 cells

Differentiated THP-1 cells were left untreated (control) or stimulated with 10ng/ml IL-33 (24hr). Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against phosphorylated-c-Jun and total-c-Jun. Panel A = Representative blot and Panel B = Densitometry graph. The image presented is representative of 3 experimental repeats. The graph displays the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test. *P= <0.05.

Figure 5.14 shows that 24hrs of IL-33 stimulation (10ng/ml) produced an increased level of phosphorylation to c-Jun, suggesting that it is activated after IL-33 stimulation. The expression level of total c-Jun in differentiated THP-1 cells was unchanged after IL-33 stimulation (Figure 5.14).

5.3.3.4 – Akt

Akt is one of the downstream mediators of PI3K activation (Heinonen et al. 2008). In order to determine the phosphorylation state of Akt after IL-33 stimulation, an antibody was used that detects phosphorylation of Akt at Ser473. Differentiated THP-1 cells were stimulated for 24hrs with 10ng/ml IL-33. The 24hr duration of stimulation was chosen on the basis of the response presented in Chapter 3. The resulting western blot is presented in Figure 5.15.

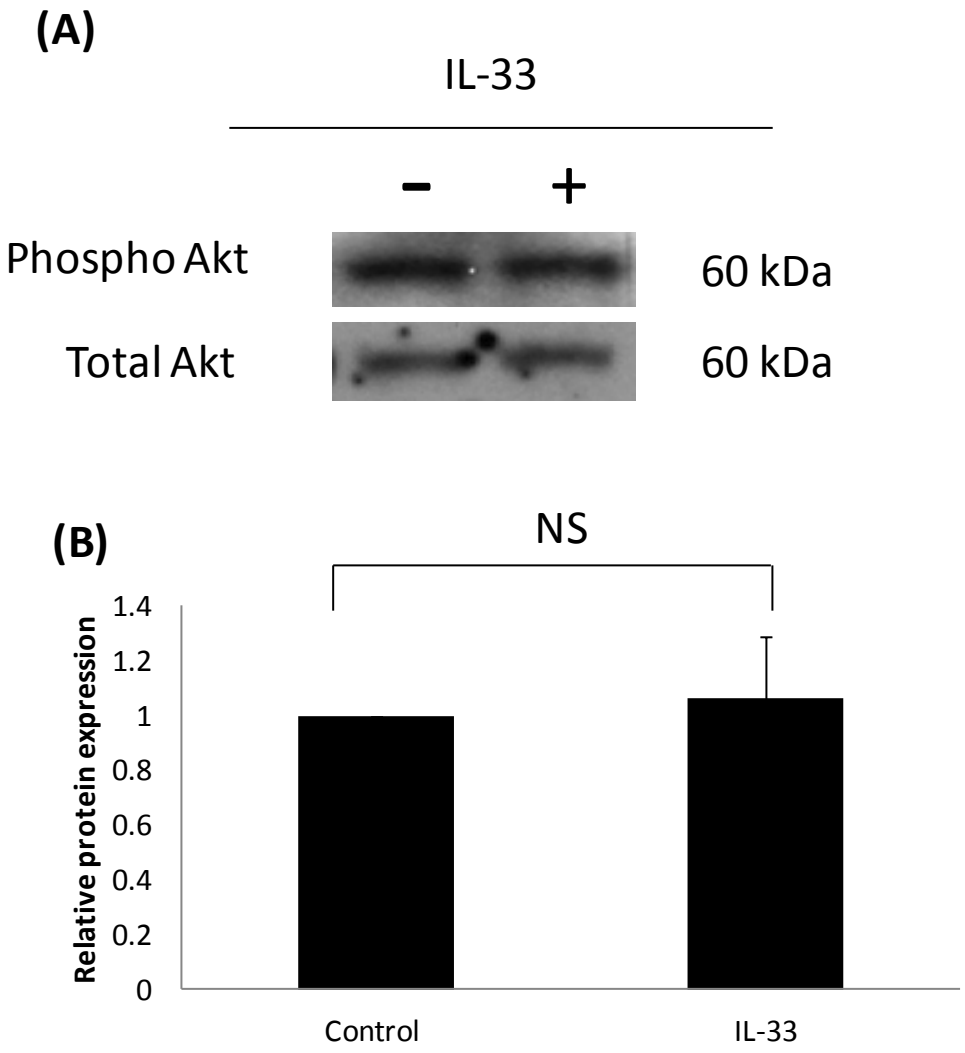


Figure 5.15: Akt was not activated in response to IL-33 stimulation of differentiated THP-1 cells

Differentiated THP-1 cells were left untreated (control) or stimulated with 10ng/ml IL-33 (24hr). Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against phosphorylated-Akt and total-Akt. Panel A = Representative blot and Panel B = Densitometry graph. The image presented is representative of 3 experimental repeats. The graph displays the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performed using the Student's t-test.

Figure 5.15 shows that 24hrs of IL-33 stimulation (10ng/ml) produced no significant change to the level of phosphorylation to Akt compared to the control, suggesting that

Akt was not activated. The expression level of total Akt in differentiated THP-1 cells was unchanged after IL-33 stimulation (Figure 5.15).

5.3.3.5 – NFκB activation

In order to ascertain whether NFκB was activated by IL-33 stimulation of macrophages, a luciferase reporter vector containing an insert of four NFκB consensus enhancer elements (pNFκB-Luc) was used for transfection based studies in RAW264.7 cells. The RAW264.7 cells were co-transfected with another expression vector that encodes the human IκBα protein (IκB-SR) which acts to inhibit NFκB signalling when over expressed. The inclusion of IκB-SR acted as a positive control for the reduction of NFκB activity within this experimental system. The IκBα cDNA was cloned into the expression plasmid pcDNA3, therefore the empty expression plasmid was used as controls within the investigation. The results from the experiment are shown in Figure 5.16.

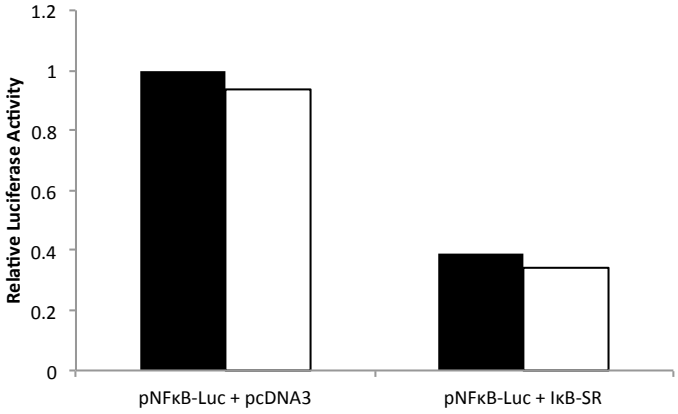


Figure 5.16: NFκB was not activated by mIL-33 stimulation of RAW264.7 cells

RAW264.7 cells were transiently co-transfected with 2μg of pNFκB-Luc plasmid and 2μg of IκB-SR or pcDNA3 using SuperFect™. The cells were then either left untreated (filled bars) or stimulated with 10ng/ml mIL-33 for 24hrs (open bars). The cells were then harvested and the resulting cell lysates were analysed for luciferase activity. Relative luciferase activity (RLA) readings were normalised to protein concentration as determined by the BCA protein assay (values from untreated samples arbitrarily assigned as 1), from 1 experiment.

Figure 5.16 suggests that when RAW264.7 cells were stimulated with mIL-33 the NFκB pathway was not activated. The data is from only one experiment so statistical analysis

cannot be carried out on the results. As expected, the over expression of IκBα by the plasmid caused a reduction in the activity through NFκB, which provides a robust positive control for the experiment.

When using a transcription based system to study regulation of gene transcription, it is possible to use specific techniques to delineate the intracellular mechanism behind the regulation. This is achieved by using dominant-negative plasmids or super-repressor plasmids of specific signalling cascades.

A co-transfection experiment was utilised to determine if NFκB was involved in the transduction of IL-33s regulation over ADAMTS-4. The ADAMTS-4 deletion construct (p-789-Luc) was transfected along with IκB-SR into RAW264.7 cells. The cells were then stimulated with mIL-33 for 24hrs. The results from this experiment are presented in Figure 5.17.

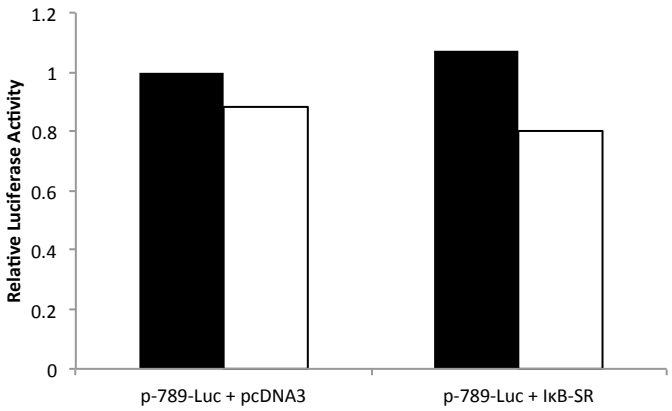


Figure 5.17: NFκB was not involved in the reduction of ADAMTS-4 promoter activity by mIL-33 stimulation of RAW264.7 cells

RAW264.7 cells were transiently co-transfected with 2µg of p-789-Luc plasmid and 2µg of IκB-SR or pcDNA3 (control) using SuperFect™. The cells were then either left untreated (filled bars) or stimulated with 10ng/ml mIL-33 for 24hrs (open bars). The cells were then harvested and the resulting cell lysates were analysed for luciferase activity. Relative luciferase activity (RLA) readings were normalised to protein concentration as determined by the BCA protein assay (values from untreated samples arbitrarily assigned as 1), from 1 experiment.

Figure 5.17 showed that when RAW264.7 cells were stimulated with mIL-33 ADAMTS-4 promoter activity was reduced slightly, confirming what was previously shown in Figure 5.10. When the cells are also transfected with a suppressor of NFκB activity the activity of the ADAMTS-4 was again slightly reduced by mIL-33 stimulation. These data suggests that the NFκB pathway was not involved in transducing the reduction of ADAMTS-4 promoter activity by IL-33.

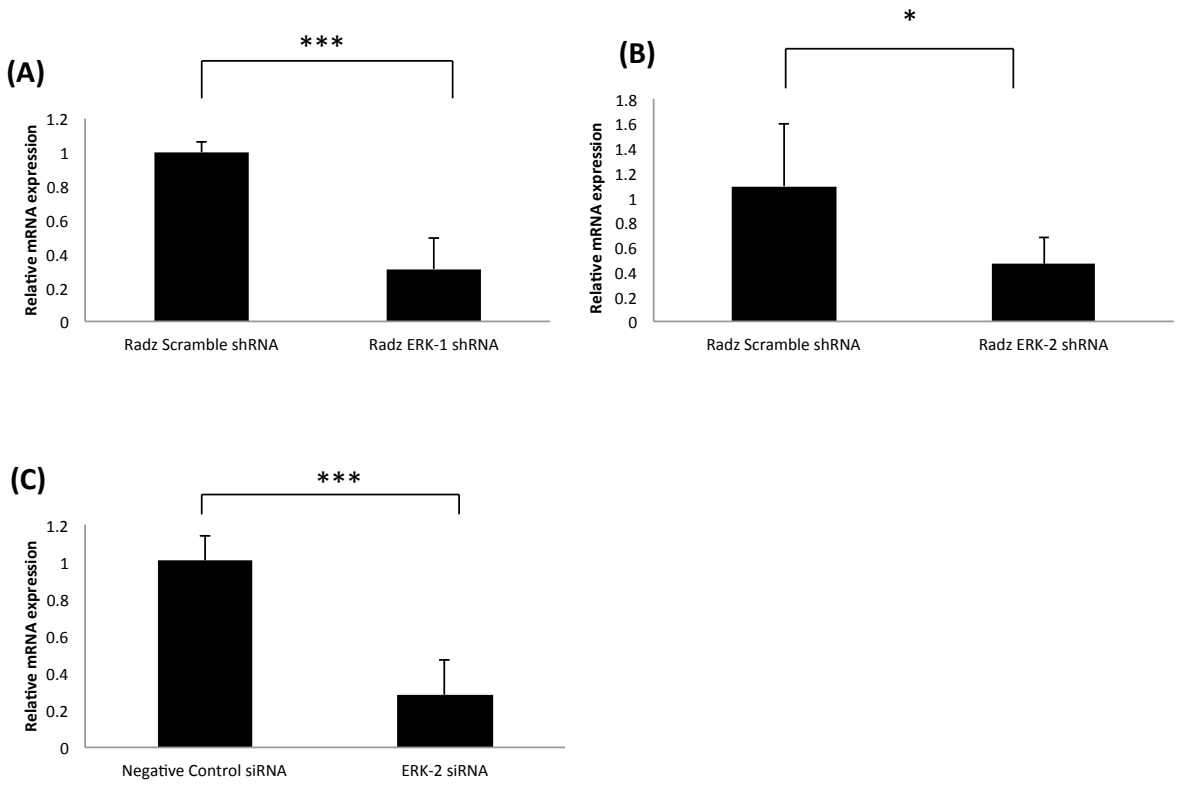
When analysing the data that had been obtained from the transfection experiments, it was observed that the changes in ADAMTS-4 promoter activity were significant, yet small (Figure 5.10). This coupled with the fact that no deletion constructs were available for ADAMTS-1 and -5 suggested the need for a new approach to be designed to delineate the mechanism behind the IL-33 response over ADAMTS-1, -4 and -5. RNAi was chosen to target key components of specific signalling cascades that have previously been linked with IL-33 signal transduction (Table 5.1 and 5.2). RNAi is a very specific gene silencing technique that avoids some potential off-target effects that are sometimes attributed to pharmacological inhibitors (Eggert et al. 2006).

5.3.4 – RNAi and IL-33 stimulation

Due to a problem with Q-PCR variability with ADAMTS-5, the mechanistic studies presented within this chapter focus on ADAMTS-1 and -4. The investigation into the mechanism behind IL-33 regulation of ADAMTS-5 had to be suspended. The reason for the variation observed within the ADAMTS-5 QPCR is largely unknown but it could be attributed to lower gene expression levels in THP-1 cells, compared to that of ADAMTS-1 and -4.

5.3.4.1 – ERK-1 and -2

ERK-1 and ERK-2 are key components of the ERK MAPK signalling pathway, so they were chosen as RNAi target genes (Dhillon et al. 2007). Firstly, the level of gene silencing produced by RNAi was studied using QPCR. These experiments were carried out in order to validate the action of the shRNA and siRNA targeting ERK-1 and -2 (Figure 5.18).



<u>Figure 5.18:</u> ERK-1 and -2 shRNA and siRNA action reduced the expression of ERK-1 and -2 mRNA in differentiated THP-1 cells

Differentiated THP-1 cells were infected with an adenovirus expressing a scramble control, ERK-1 or ERK-2 shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ERK-1 and B = ERK-2. Similar experiments were carried out that utilised siRNA to target a scrambled sequence or ERK-2. Panel C = ERK-2. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, ***P=<0.001.

The reduction of ERK-1 mRNA expression caused by the shRNA was validated using Q-PCR. It was shown that the ERK-1 shRNA reduced mRNA expression levels by 69% (Figure 5.18, panel A). Also, the specificity of the ERK-1 shRNA was checked by analysing ERK-2 mRNA expression, the expression of ERK-2 mRNA was unchanged (Figure 5.19, panel B). ERK-2 shRNA caused a reduction in mRNA expression by 54% (Figure 5.18, panel B); the reduction was also shown to be specific, as ERK-1 expression was not changed by the ERK-2 shRNA (Figure 5.19, panel A). Following on from this, the siRNA mediated

knockdown of ERK-2 resulted in a 72% reduction in the mRNA expression (Figure 5.18, Panel C). Again the expression of ERK-1 mRNA was unchanged which highlighted the specificity of the siRNA (Figure 5.19, Panel C).

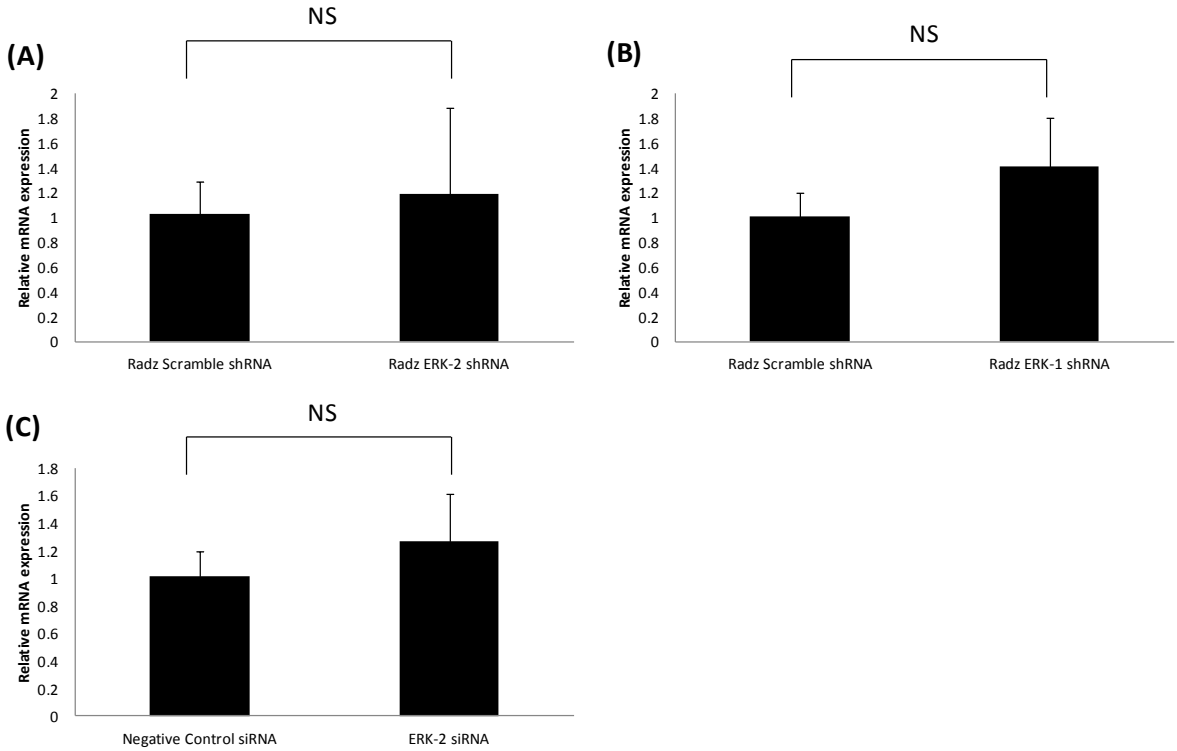


Figure 5.19: ERK-1 and -2 shRNA and siRNA action was specific to the target mRNA in differentiated THP-1 cells

Differentiated THP-1 cells were infected with an adenovirus expressing a scramble control, ERK-1 or ERK-2 shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ERK-1 and B = ERK-2. Similar experiments were carried out that utilised siRNA to target a scrambled sequence or ERK-2. Panel C = ERK-1. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test.

After the Q-PCR validation experiments had been carried out representative samples were produced and analysed using SDS-PAGE and western blot to validate the gene silencing action at the protein level (Figure 5.20 and 5.21).

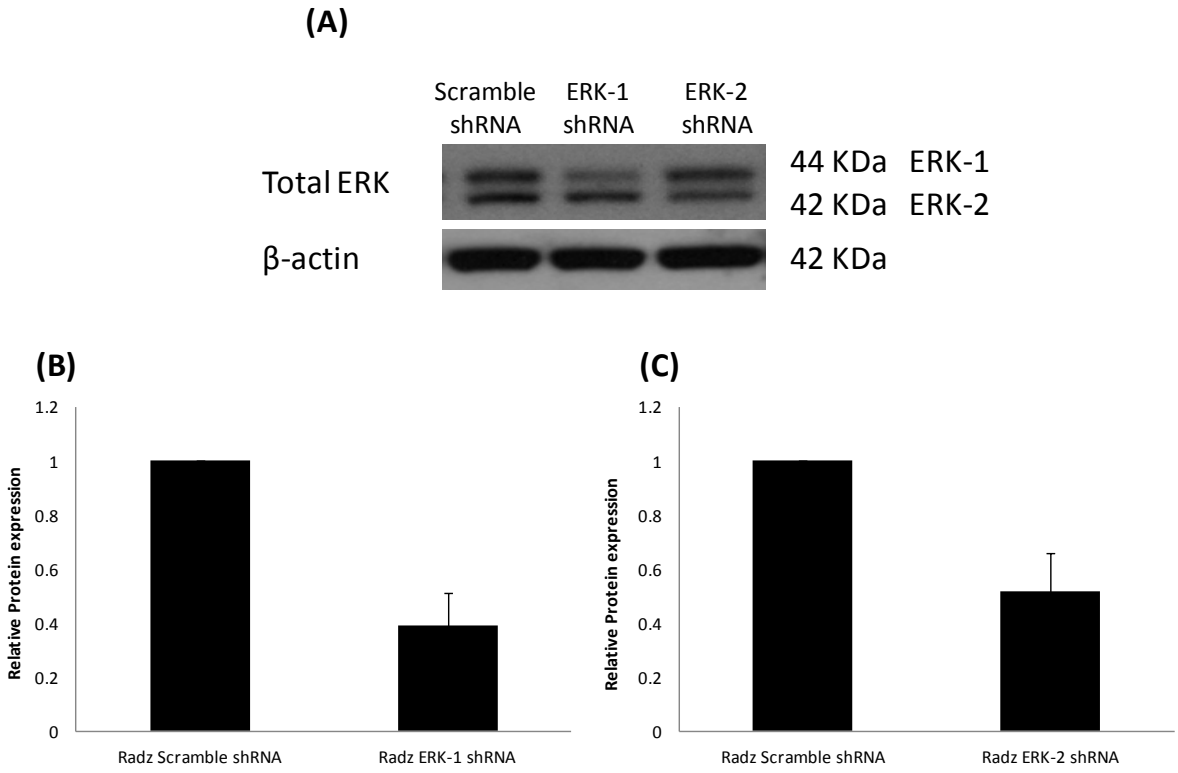


Figure 5.20: ERK-1 and -2 shRNA reduced the expression of ERK-1 and -2 protein in differentiated THP-1 cells

Differentiated THP-1 cells were infected with adenoviruses expressing a scrambled sequence (control), ERK-1 shRNA or ERK-2 shRNA. Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against total-ERK and β-actin. Panel A = Representative blot. The blot presented is representative of 2 experimental repeats. Panel B = ERK-1 shRNA densitometry graph and Panel C = ERK-2 shRNA densitometry graph. The graphs display the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 2 independent experimental repeats.

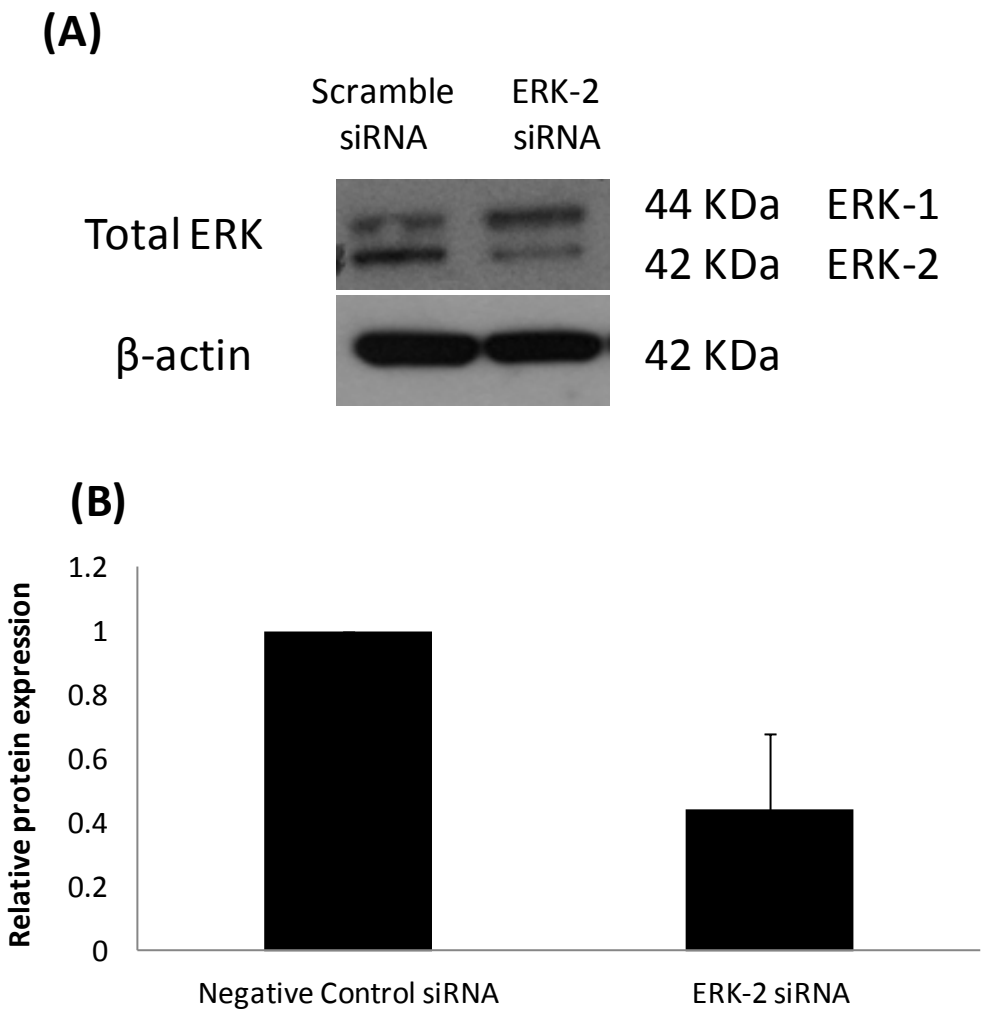


Figure 5.21: ERK-2 siRNA action reduced the expression of ERK-2 protein in differentiated THP-1 cells

Differentiated THP-1 cells were transfected with siRNA targeting a scrambled sequence (negative control), or ERK-2. Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against total-ERK and β-actin. Panel A = Representative blot. The blot presented is representative of 2 experimental repeats. Panel B = ERK-2 siRNA densitometry graph. The graph displays the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 2 independent experimental repeats.

It was observed that when ERK-1 and -2 shRNAs were expressed within differentiated THP-1 cells, reductions in ERK-1 and -2 protein expressions were detected (Figure 5.20).

Also, after transfection with a siRNA targeting ERK-2, the expression of ERK-2 protein was reduced in differentiated THP-1 cells (Figure 5.21).

Following on from the protein validation experiments, Q-PCR was utilised to study if a functional reduction of ERK-1 and -2 expressions could alter how IL-33 regulated ADAMTS-1 and -4 expressions (Figure 5.22, 5.23 and 5.24).

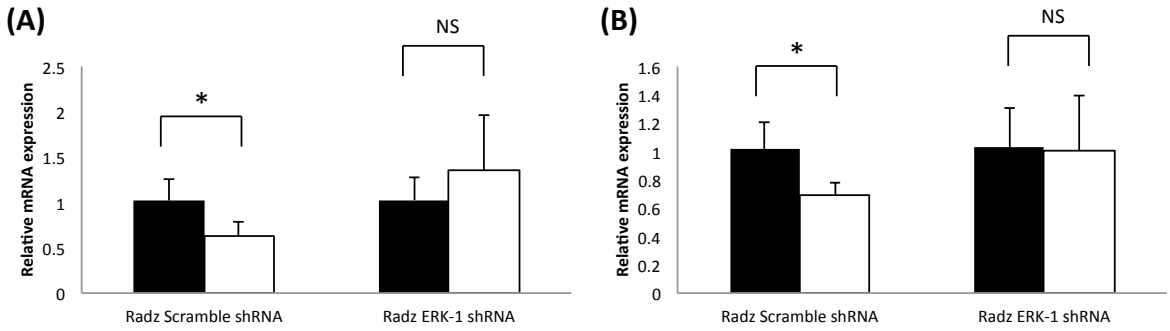


Figure 5.22: IL-33 regulated the expression of ADAMTS-1 and -4 through ERK-1

Differentiated THP-1 cells were infected with an adenovirus expressing either a scramble control or ERK-1 shRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1 and B = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05.

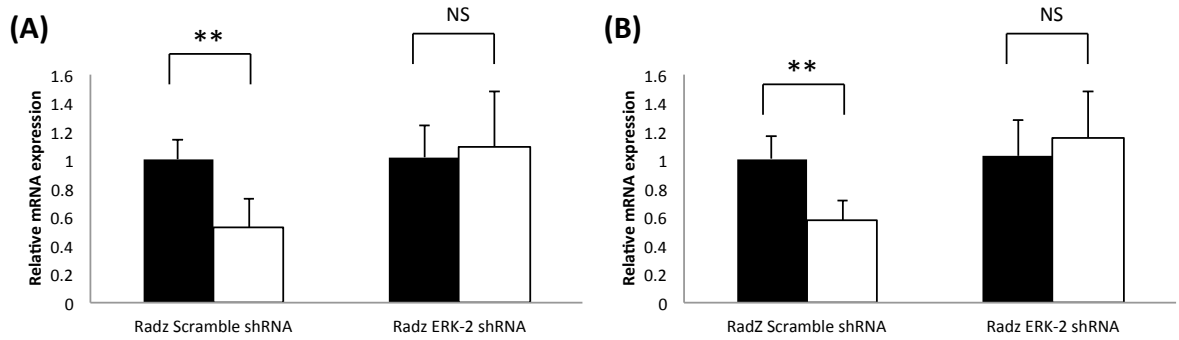


Figure 5.23: IL-33 regulated the expression of ADAMTS-1 and -4 through ERK-2

Differentiated THP-1 cells were infected with an adenovirus expressing either a scramble control or ERK-2 shRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1 and B = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. **P=<0.01.

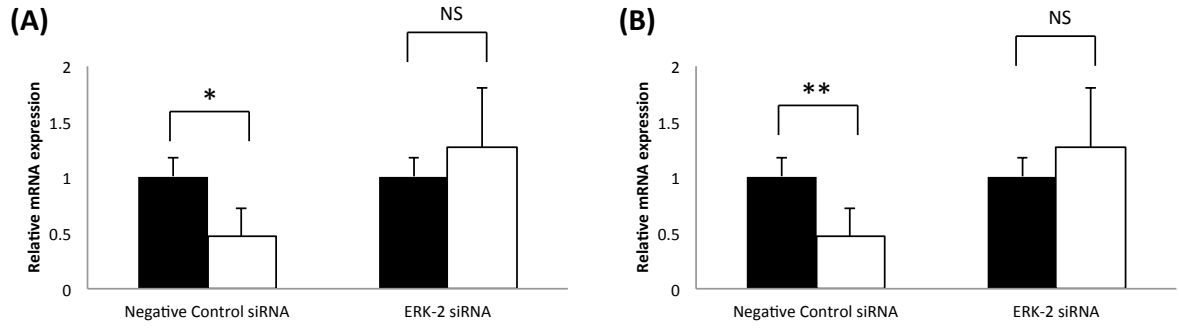


Figure 5.24: IL-33 regulated the expression of ADAMTS-1 and -4 through ERK-2

Differentiated THP-1 cells were transfected with a siRNA targeting either a scramble control or ERK-2. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1 and B = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, **P=<0.01.

The IL-33-mediated reduction in ADAMTS-1 and -4 expressions seen when the cells were infected with a scrambled shRNA sequence was attenuated/lost when the cells were infected with virus encoding ERK-1 and -2 shRNA sequences (Figures 5.22 and 5.23).

Further experiments were carried that utilised siRNA to reduce the expression of ERK-2 in THP-1 cells, followed by IL-33 stimulation. These experiments acted as an independent confirmation that ERK-2 had an important role in the transduction of IL-33s regulation over ADAMTS-1 and -4 (Figure 5.24).

5.3.4.2 – p38α shRNA

p38α is the most abundant isoform of p38 found within inflammatory cells and has been widely studied (Chung 2011). For these reasons it was identified as a target molecule for RNAi. Firstly, the level of gene silencing produced by RNAi was studied using QPCR. These experiments were carried out in order to validate the action of the shRNA targeting p38α (Figure 5.25).

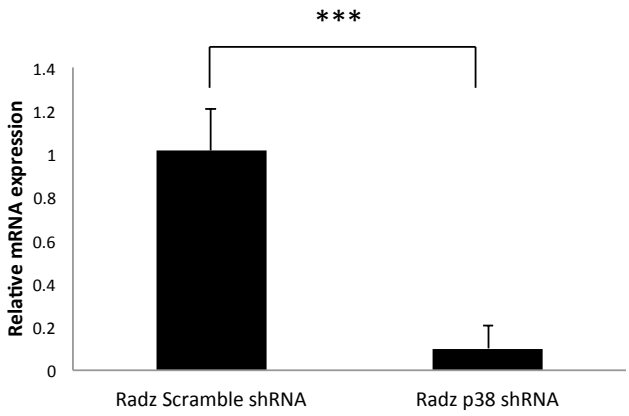


Figure 5.25: p38α shRNA action reduced the expression of p38α mRNA in differentiated THP-1 cells

Differentiated THP-1 cells were infected with an adenovirus expressing a scramble control or p38α shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. The graph displays the average fold change in p38α mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. ***P=<0.001.

The reduction of p38α mRNA expression caused by the shRNA was validated by Q-PCR within the experimental samples. It was shown that the p38α shRNA significantly reduced the mRNA expression levels by 90% (Figure 5.25).

After the Q-PCR experiments had been carried out, representative samples were produced and analysed using SDS-PAGE and western blot (Figure 5.26).

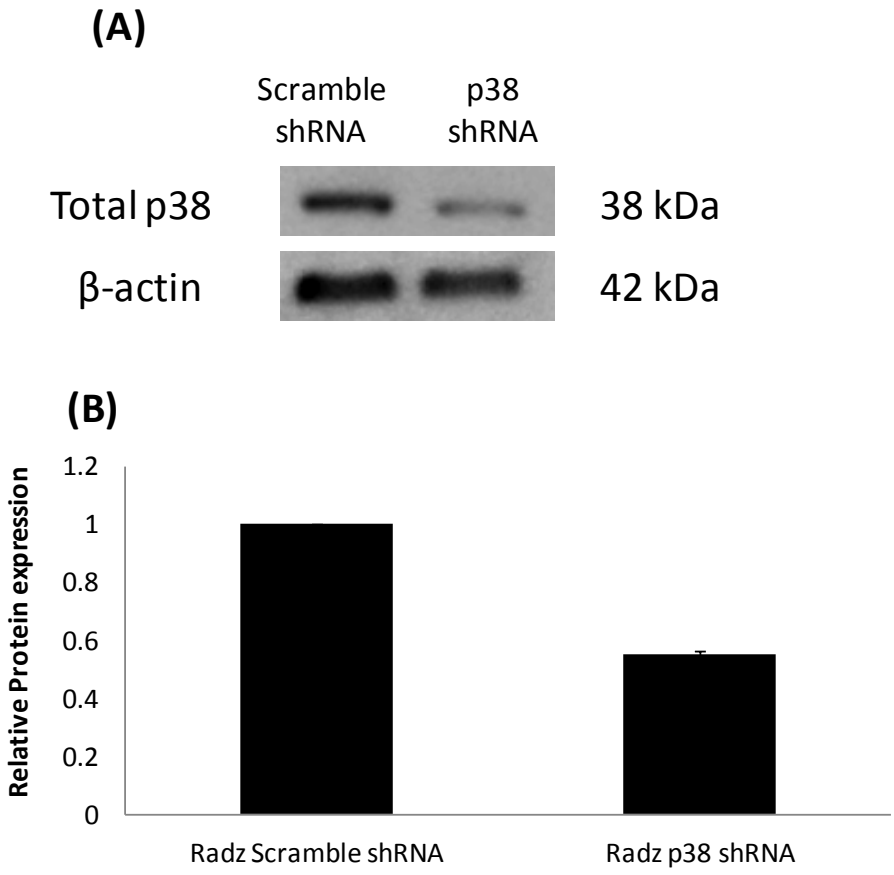


Figure 5.26: p38α shRNA action reduced the expression of p38α protein in differentiated THP-1 cells

Differentiated THP-1 cells were infected with adenoviruses expressing a scrambled sequence (control) or p38α shRNA. Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against total-p38α and β-actin. Panel A = Representative blot and Panel B = p38α shRNA densitometry graph. The graph displays the average fold change in protein expression in relation to the control (arbitrarily assigned as 1), from 2 independent experimental repeats. The blot presented is representative of the 2 experimental repeats.

After the differentiated THP-1 cells had been infected with an adenovirus that caused expression of a shRNA targeting p38α, the protein level of p38α was reduced (Figure 5.26).

Following on from the protein validation experiments, Q-PCR was utilised to study if a functional reduction of p38α expression could alter how IL-33 regulated ADAMTS-1 and -4 expressions (Figure 5.27).

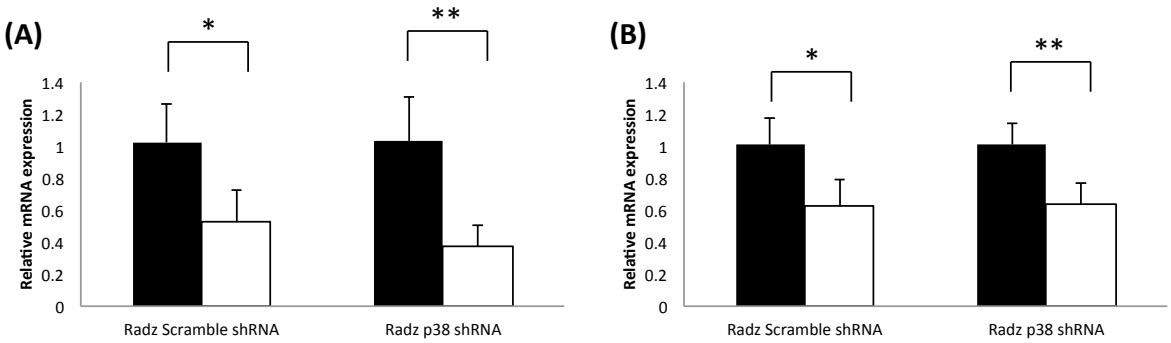


Figure 5.27: IL-33 did not regulate the expression of ADAMTS-1 and -4 through p38α

Differentiated THP-1 cells were infected with an adenovirus expressing either a scramble control or p38α shRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = p38α. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, ***P=<0.001.

The IL-33-mediated reduction in ADAMTS-1 and -4 expressions seen when the cells were infected with a scrambled shRNA sequence was not affected when the cells were infected with virus encoding p38α shRNA sequences (Figures 5.27).

5.3.4.3 – JNK-1/2 siRNA

JNK-1 and JNK-2 are key components of the JNK MAPK signalling pathway, so they were chosen as RNAi target genes (Dhillon et al. 2007). Transfection of chemically synthesised siRNAs targeting JNK-1 and -2 mRNA expressions were used in differentiated THP-1 cells. Unfortunately a problem was encountered with antibody detection during the studies. In this case the reduction of JNK-1 and -2 mRNA expressions was used for siRNA validation. JNK-1 and -2 mRNA expressions were significantly reduced by siRNA transfection by 54% and 45% respectively (Figure 5.28, panels A and B). Following on from the validation of the siRNA, Q-PCR was utilised to study if a reduction of JNK-1 and -2 expressions could alter how IL-33 regulates ADAMTS-1 and -4 expressions (Figure 5.28, panels C and D).

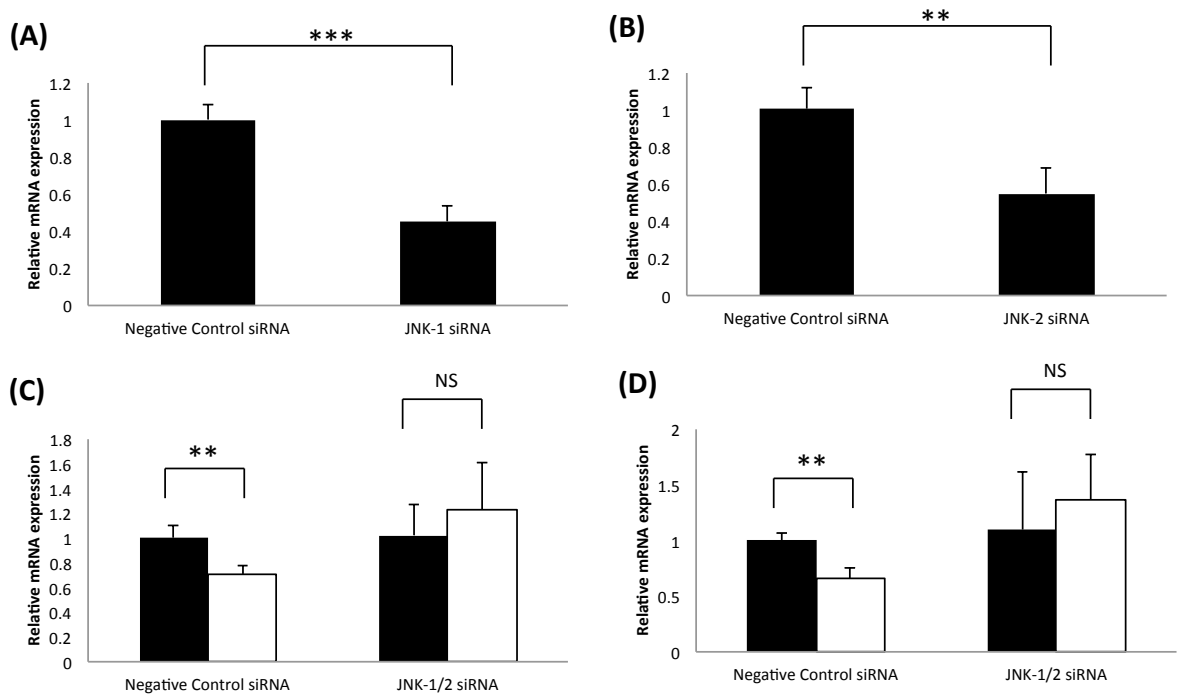


Figure 5.28: IL-33 regulated the expression of ADAMTS-1 and -4 through JNK-1 and -2

Differentiated THP-1 cells were transfected with either a negative control siRNA or JNK-1/2 siRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = JNK-1, B = JNK-2, C = ADAMTS-1 and D = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, **P=<0.01, ***P=<0.001.

The IL-33-mediated reduction in ADAMTS-1 and -4 expressions seen when the cells were transfected with a scrambled siRNA sequence was attenuated/lost when the cells were transfected with JNK-1 and -2 siRNA (Figures 5.28).

5.3.4.4 – c-Jun siRNA

As mentioned previously, c-Jun is a transcription factor that is a downstream target of JNK within the MAPK signalling cascade (Kim and Choi 2010). For this reason it was chosen as a target for RNAi. Firstly, the level of gene silencing produced by RNAi was

studied using QPCR. These experiments were carried out in order to validate the action of the siRNA targeting c-Jun (Figure 5.29).

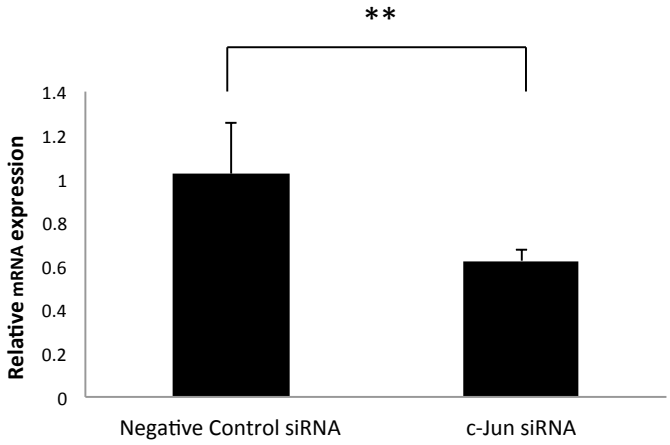


Figure 5.29: c-Jun siRNA action reduced the expression of c-Jun mRNA in differentiated THP-1 cells

Differentiated THP-1 cells were transfected with a scramble control or c-Jun siRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. The graph displays the average fold change in c-Jun mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. **P=<0.01.

The reduction of c-Jun mRNA expression caused by the siRNA was validated by Q-PCR within the experimental samples. It was shown that the c-Jun siRNA significantly reduced the mRNA expression levels by 38% (Figure 5.29).

After the Q-PCR experiments had been carried out representative samples were produced and analysed using SDS-PAGE and western blot analysis (Figure 5.30).

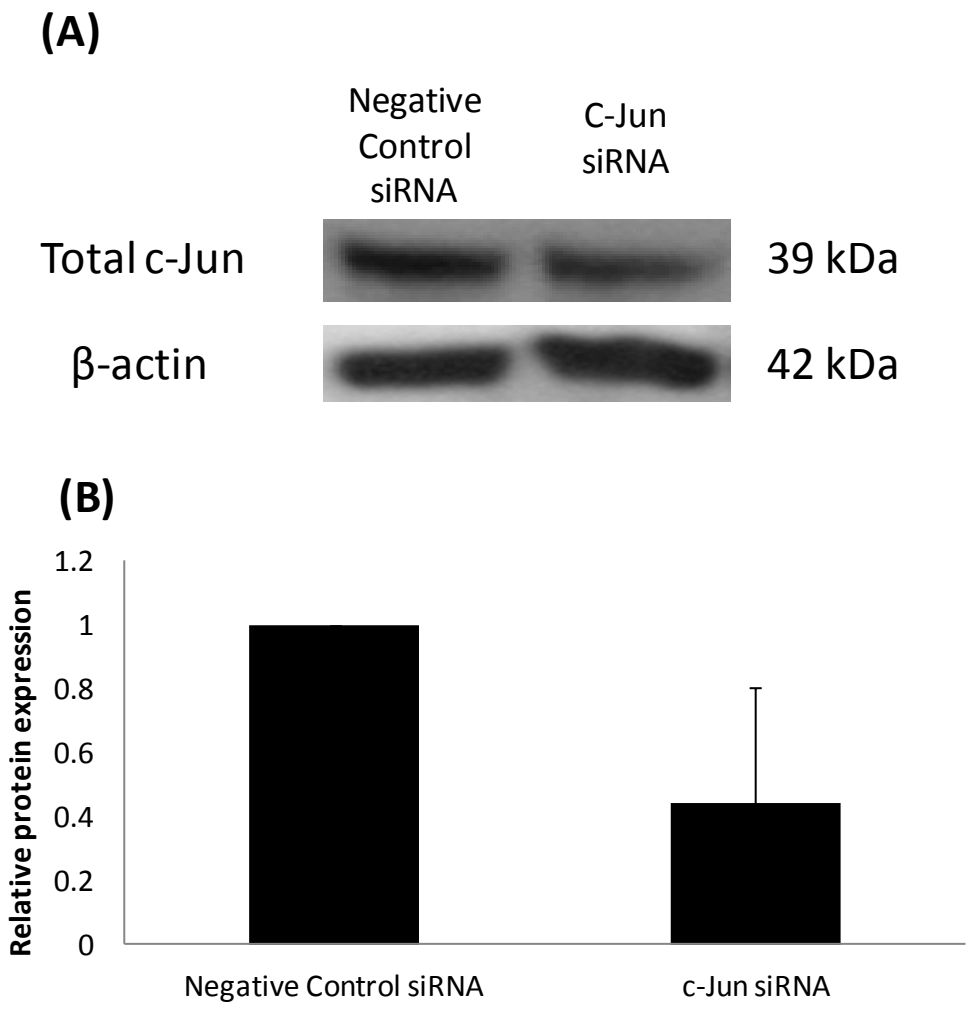


Figure 5.30: c-Jun siRNA action reduced the expression of c-Jun protein in differentiated THP-1 cells

Differentiated THP-1 cells were transfected with a negative control siRNA or a c-Jun siRNA. Whole cell lysates were subjected to SDS-PAGE and western blotting using antibodies against total-c-Jun and β-actin. Panel A = Representative blot and Panel B = c-Jun siRNA densitometry graph. The graph displays the average fold change in protein expression in relation to the control, from 2 independent experimental repeats. The blot presented is representative of 2 experimental repeats.

After the differentiated THP-1 cells had been transfected with a siRNA targeting c-Jun, the protein level was reduced (Figure 5.30). Following on from the validation experiments, Q-PCR was utilised to study if a functional reduction of c-Jun expression could alter how IL-33 regulated ADAMTS-1 and -4 expressions (Figure 5.31).

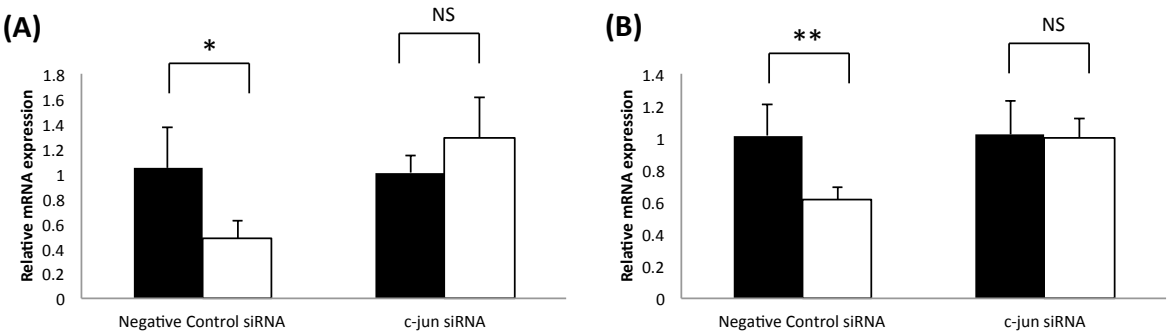


Figure 5.31: IL-33 regulated the expression of ADAMTS-1 and -4 through c-Jun

Differentiated THP-1 cells were transfected with either a negative control siRNA or c-Jun siRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1 and B = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, **P=<0.01.

The IL-33-mediated reduction in ADAMTS-1 and -4 expressions seen when the cells were transfected with a scrambled siRNA sequence was attenuated/lost when the cells were transfected with c-Jun siRNA (Figures 5.31).

5.3.4.5 – PI3Kγ shRNA

PI3K p110-γ and δ have been shown to be expressed by haematopoietic cells and they are activated downstream of G-protein coupled receptors and RTKs (Williams *et al.* 2010b). They have been suggested to regulate immune cell signalling pathways (Williams et al. 2010b). The redundant roles of PI3Kγ and δ have made it difficult to determine which is best to target for therapeutic interventions for many inflammatory conditions (Williams et al. 2010b). Here, PI3Kγ was initially targeted by RNAi, followed by PI3Kδ in the next section (5.3.4.6) to identify its role in the IL-33 induced reduction of ADAMTS-1 and -4 expressions. Adenoviral delivery of a shRNA targeting PI3Kγ mRNA expression was used in differentiated THP-1 cells. Unfortunately an antibody was not available in our laboratory to validate the reduction of PI3Kγ expression. In this case the reduction of

PI3Kγ mRNA expression was used for shRNA validation. PI3Kγ mRNA expression was significantly reduced by shRNA expression by 91% (Figure 5.32, panel A). Following on from the validation of the shRNA, Q-PCR was utilised to study if a reduction of PI3Kγ expression could alter how IL-33 regulates ADAMTS-1 and -4 expressions (Figure 5.32).

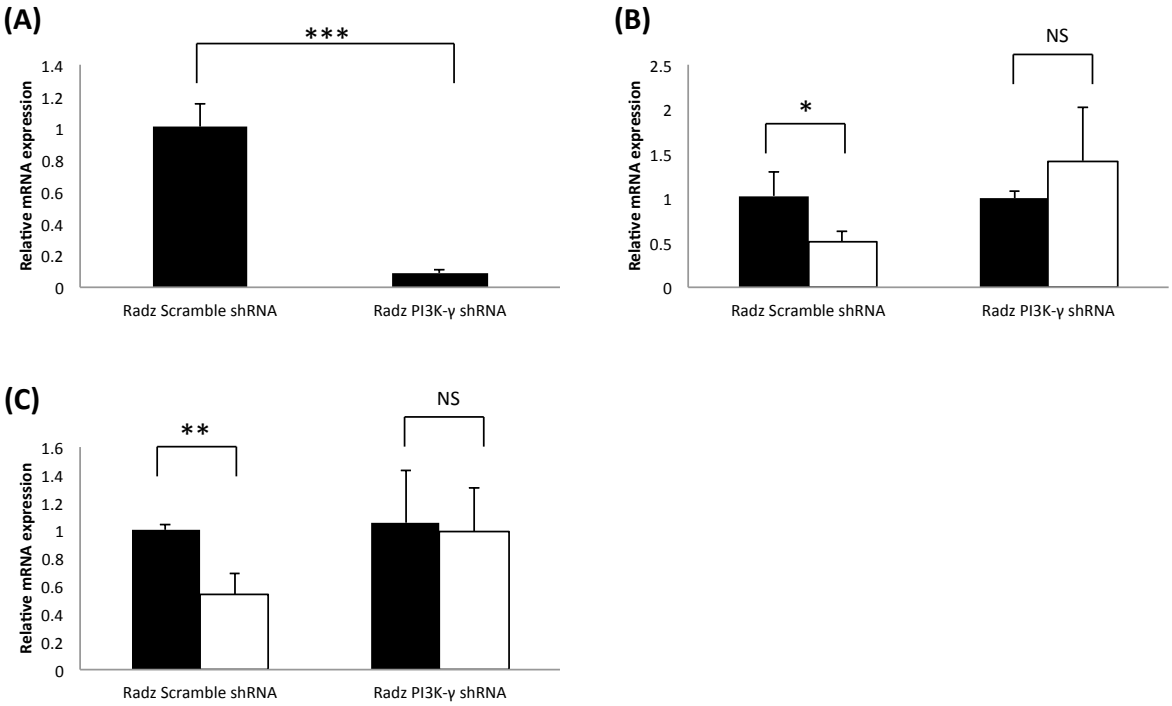


Figure 5.32: IL-33 regulated the expression of ADAMTS-1 and -4 through PI3Kγ

Differentiated THP-1 cells were infected with an adenovirus expressing either a scramble control or PI3Kγ shRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = PI3Kγ, B = ADAMTS-1 and C = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, **P=<0.01, ***P=<0.001.

The IL-33-mediated reduction in ADAMTS-1 and -4 expressions seen when the cells were infected with a scrambled shRNA sequence was attenuated/lost when the cells were infected with virus encoding PI3Kγ shRNA sequence (Figures 5.32).

5.3.4.6 – PI3Kδ shRNA

Adenoviral delivery of a shRNA targeting PI3Kδ mRNA expression was used in differentiated THP-1 cells. Again, an antibody was not available in our laboratory to validate the reduction of PI3Kδ expression. In this case the reduction of PI3Kδ mRNA expression was used for shRNA validation. PI3Kδ mRNA expression was significantly reduced by shRNA expression by 86% (Figure 5.33, panel A). Following on from the validation of the shRNA, Q-PCR was utilised to study if a reduction of PI3Kδ expression could alter how IL-33 regulates ADAMTS-1 and -4 expressions (Figure 5.33, panels B and C).

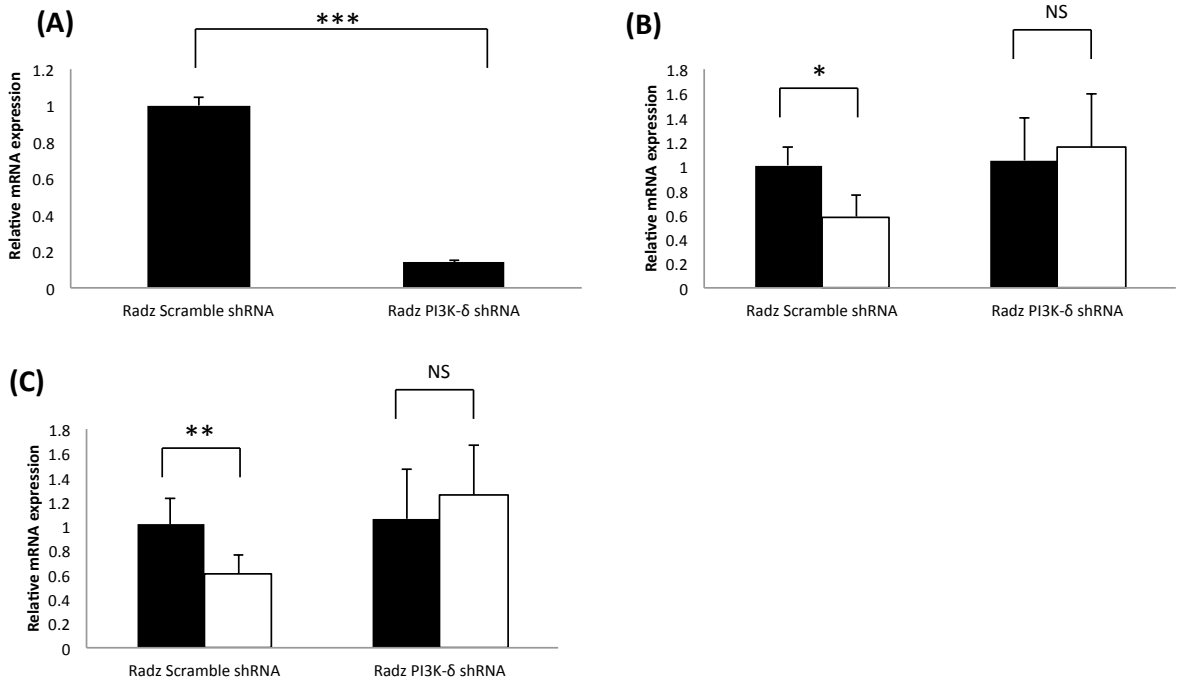


Figure 5.33: IL-33 regulated the expression of ADAMTS-1 and -4 through PI3Kδ

Differentiated THP-1 cells were infected with an adenovirus expressing either a scramble control or PI3Kδ shRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = PI3Kδ, B = ADAMTS-1 and C = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, **P=<0.01, ***P=<0.001.

The IL-33-mediated reduction in ADAMTS-1 and -4 expressions seen when the cells were infected with a scrambled shRNA sequence was attenuated/lost when the cells were infected with virus encoding PI3Kδ shRNA sequence (Figures 5.33).

5.3.4.7 – p50 and p65

p50 and p65 are members of the NFκB family of transcription factors (Pamukcu et al. 2011). The transcription factors are usually found in inactive dimers in the cytoplasm bound to IκB, which is an inhibitor of NFκB action (Pamukcu et al. 2011). There are many family members of NFκB, however p50 and p65 are the main components of the active dimer which causes regulation over gene transcription (Li and Verma 2002). As the proteins are the main components of the active dimer it was decided that they would make good targets for RNAi within our studies.

Unfortunately when the siRNA-mediated reduction of p50 and p65 gene expression was carried out in THP-1 macrophages some technical difficulties were met. After transfection with the siRNAs the amount and quality of RNA prepared from the cells was extremely variable. Due to this technical problem the investigation into the role of p50 and p65 had to be suspended.

5.3.4.8 – JAK-1 and -2

JAKs are protein kinases that are associated/activated by tyrosine kinase receptors and are involved in JAK-STAT signalling (Schindler et al. 2007). JAK-1 and JAK-2 have a wide expression profile whereas JAK-3 is limited to leukocytes (Schindler et al. 2007). It has been shown previously that JAK family inhibitors and JAK-2 inhibitors, but not JAK-3 inhibitors effectively attenuated NFκB activation after IL-33 stimulation of murine peritoneal macrophages (Funakoshi-Tago et al. 2011). For this reason JAK-1 and -2 were chosen for targets of RNAi for our studies.

Similar to the problems that were observed in the previous section, when the siRNA-mediated reduction of JAK-1 and -2 expressions was carried out upon THP-1 macrophages some technical difficulties were met. After transfection with the siRNAs the

amount and quality of RNA prepared from the cells was extremely variable. Due to this technical problem the investigation into the role of JAK-1 and -2 had to be suspended.

5.3.4.9 – RNAi and the constitutive expressions of ADAMTS-1 and -4

It was of interest to analyse how RNAi assays that targeted key components of signalling pathways were regulating the constitutive expression of ADAMTS-1 and -4 from un-stimulated differentiated THP-1 cells in response to PMA (Table 5.3).

Table 5.3: How the RNAi targets regulated ADAMTS-1 and -4 constitutive gene expressions in differentiated THP-1 cells

	Control	ERK-1	ERK-2	p38α	JNK-1 and -2	c-Jun	PI3Kγ	PI3Kδ
ADAMTS-1	1.02 ±0.2	1.06 ±0.65	1.18 ±1.18	0.43 ±0.17 ***	1.13 ±0.31	0.98 ±0.3	0.13 ±0.18 ***	1.11 ±1.04
ADAMTS-4	1.01 ±0.16	0.95 ±0.51	0.77 ±0.22	0.57 ±0.12 **	1.06 ±0.63	1.13 ±0.37	0.98 ±0.5	0.97 ±0.90

The constitutive expressions of ADAMTS-1 and 4 were analysed by QPCR after RNAi of various target molecules. Differentiated THP-1 cells were subjected to RNAi and total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. The table displays the average fold change in mRNA expression in relation to the relevant control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. **P=<0.01, ***P=<0.001.

From Table 5.3 it was observed that ADAMTS-1 constitutive gene expression was dependent on p38α and PI3Kγ, whereas ADAMTS-4 constitutive expression was dependent on p38α.

5.3.5 – Primary cell confirmation of findings in differentiated THP-1 cells

It was of interest to confirm the findings that had been observed in THP-1 cells in primary HMDMs, to this end representative RNAi experiments were carried out. Due to the low efficiency of transfection and knockdown commonly observed in HMDMs using siRNA, only RNAi that utilised viral delivery of shRNA could be performed during the current study.

The level of gene silencing produced by viral delivery of various shRNAs was studied using QPCR. These experiments were carried out in order to validate the action of the shRNAs targeting ERK-1, ERK-2, p38α, PI3Kγ and PI3Kδ (Figure 5.34).

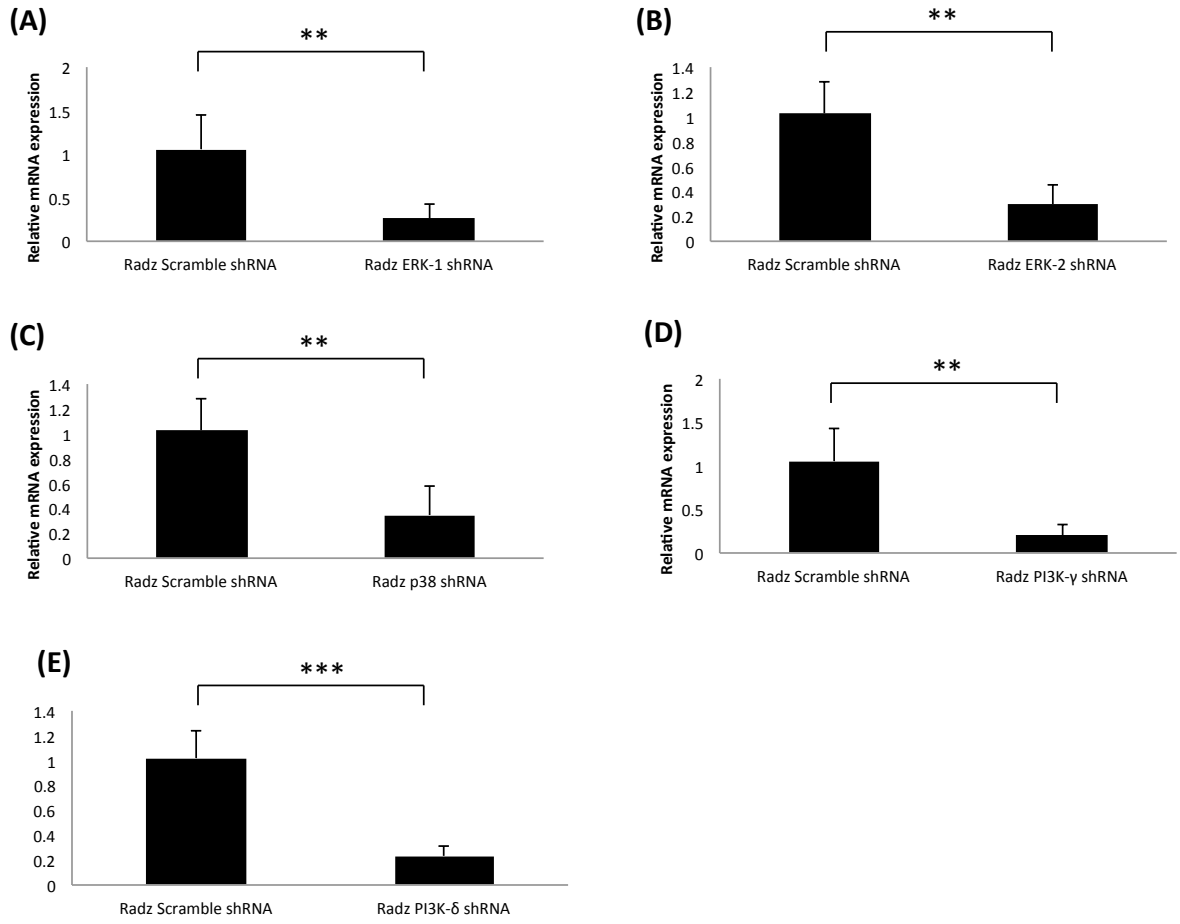


Figure 5.34: shRNA validation of mRNA knockdown in HMDMs

HMDMs were infected with an adenovirus expressing a scramble control, ERK-1, ERK-2, p38α, PI3Kγ or PI3Kδ shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ERK-1, B = ERK-2, C = p38α, D = PI3Kγ and E = PI3Kδ. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. **P=<0.01, ***P=<0.001.

The reduction of ERK-1 mRNA expression in HMDMs by shRNA was validated using Q-PCR within the experimental samples. It was shown that the ERK-1 shRNA reduced the mRNA expression levels by 74% (Figure 5.34, panel A). Also, the specificity of the ERK-1 shRNA was checked by analysing ERK-2 mRNA expression, the expression of ERK-2 mRNA was unchanged (Figure 5.35, panel B). The reduction of ERK-2 mRNA expression caused by the shRNA was confirmed in the experimental samples; a reduction of 71% was observed (Figure 5.34, panel B). The reduction was also shown to be specific, as ERK-1 expression was not changed by the ERK-2 shRNA (Figure 5.35, panel A). Following on from this, the

shRNA mediated knockdown of p38α resulted in a 66% reduction in mRNA expression (Figure 5.34, panel C). The mRNA expression of PI3Kγ was reduced by 81% and the expression of PI3Kδ was reduced by 77% after shRNA treatment of HMDMs (Figure 5.34, panels D and E).

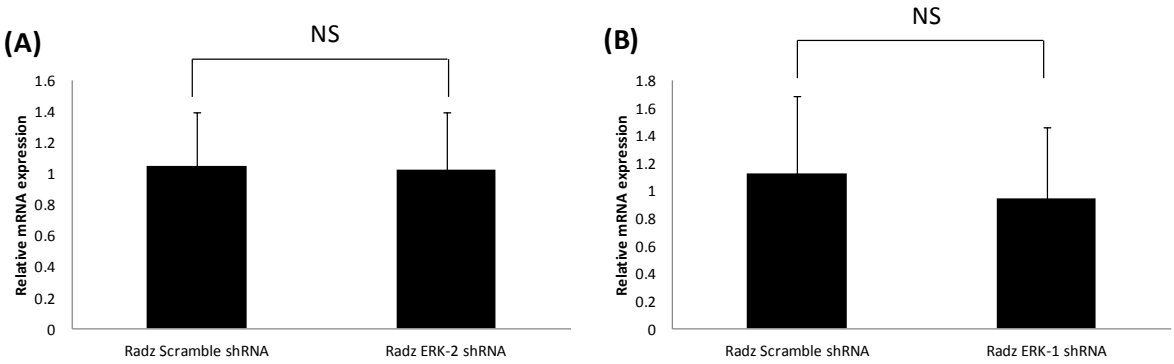


Figure 5.35: ERK-1 and -2 shRNAs were specific to the target mRNA in HMDMs

HMDMs were infected with an adenovirus expressing a scramble control, ERK-1 or ERK-2 shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ERK-1 and B = ERK-2. The graphs display the average fold change in mRNA expression in relation to the control (arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test.

Following on from the validation experiments, Q-PCR was utilised to study if a functional reduction of ERK-1, ERK-2, p38α, PI3Kγ or PI3Kδ expressions could alter how IL-33 regulated ADAMTS-1 and -4 expressions in HMDMs (Figures 5.36 and 5.37).

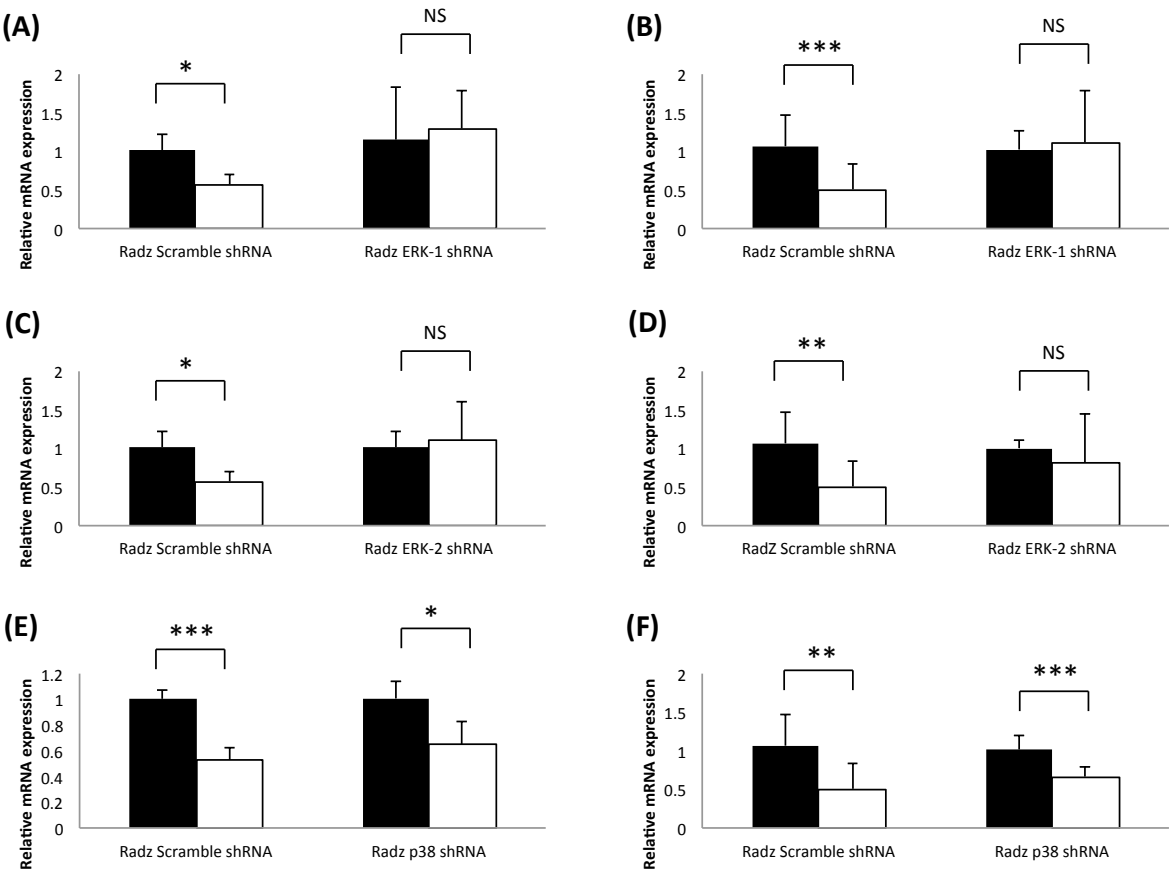


Figure 5.36: IL-33 regulated the expression of ADAMTS-1 and -4 through ERK-1 and ERK-2 but not p38α in HMDMs

HMDMs were infected with an adenovirus expressing either a scramble control, ERK-1, ERK-2 or p38α shRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panels A, C and E = ADAMTS-1 and Panels B, D and F = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, **P=<0.01, ***P=<0.001.

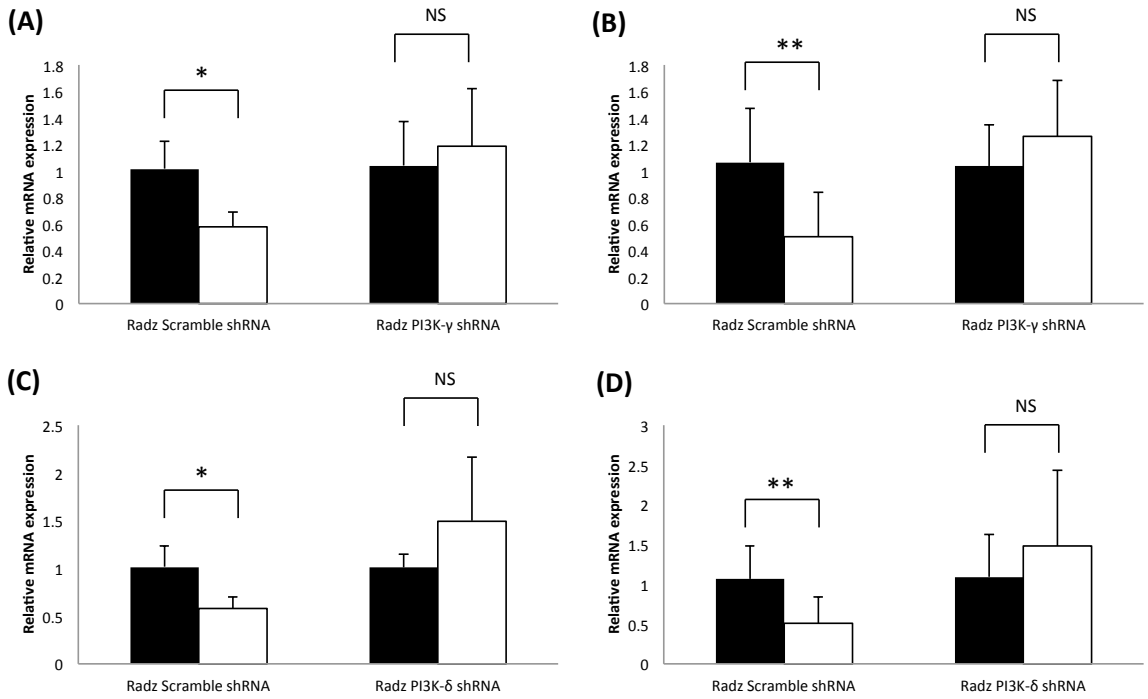


Figure 5.37: IL-33 regulated the expression of ADAMTS-1 and -4 through PI3Kγ and PI3Kδ in HMDMs

HMDMs were infected with an adenovirus expressing either a scramble control, PI3Kγ or PI3Kδ shRNA. These were then either left untreated (filled bars) or stimulated with 10ng/ml IL-33 for 24hrs (open bars). Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panels A and C = ADAMTS-1 and Panels B and D = ADAMTS-4. The graphs display the average fold change in mRNA expression in relation to the control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05, **P=<0.01.

From Figures 5.36 and 5.37 it was observed that the responses that were shown in THP-1 were conserved in HMDMs. It was of interest to elucidate if the same regulation over ADAMTS-1 and -4 constitutive expressions were conserved between THP-1 and HMDMs (Table 5.4).

Table 5.4: How the RNAi targets regulated ADAMTS-1 and -4 constitutive gene expressions in HMDMs

	Scramble Control	ERK-1	ERK-2	p38α	PI3Kγ	PI3Kδ
ADAMTS-1	1.02 ±0.21	0.84 ±0.58	0.56 ±0.51	0.49 ±0.36 *	0.65 ±0.43	1.16 ±0.84
ADAMTS-4	1.07 ±0.41	0.81 ±0.19	0.75 ±0.52	0.42 ±0.34 *	0.86 ±0.49	0.79 ±0.8

The constitutive expressions of ADAMTS-1 and -4 were analysed by QPCR after RNAi of various target molecules. HMDMs were subjected to RNAi and total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. The table displays the average fold change in mRNA expression in relation to the scramble control (control values from untreated samples arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. *P=<0.05.

It was observed that ADAMTS-1 and -4 constitutive expressions were dependent on p38α in HMDMs (Table 5.4).

5.4 – Discussion

The principal aim of this chapter was to expand on findings that were presented in Chapter 3. An important novel finding was that IL-33 stimulation of macrophages was shown to significantly reduce the expressions of ADAMTS-1, -4 and -5. It was of interest to investigate for the first time how this attenuation of gene expression was transduced within human macrophages.

Summaries of the findings presented within this chapter are as follows:

- IL-33 stimulation reduced the promoter activity of ADAMTS-4.
- The ST2 receptor was involved in the transduction of IL-33 regulation over ADAMTS-1 and -4.
- ERK-1/2 and c-Jun were activated by IL-33 stimulation of THP-1 macrophages.
- p38, Akt and NFκB were not activated by IL-33 stimulation of macrophages.
- ERK-1, ERK-2, JNK, c-Jun, PI3Kγ and PI3Kδ were involved in the transduction of IL-33s regulation over ADAMTS-1 and -4 expressions.
- p38α was not involved in the transduction of IL-33s regulation over ADAMTS-1 and -4 expressions.
- The findings presented in differentiated THP-1 cells were conserved in HMDM cells (apart from the control over ADAMTS-1 constitutive expression by PI3Kγ in THP-1 cells).

5.4.1 – IL-33 stimulation reduces ADAMTS-4 promoter activity

In Chapter 3, it was shown that IL-33 stimulation of THP-1 macrophages and HMDMs resulted in a significant reduction of ADAMTS-1 and -4 expressions. This was consistent with the protective role suggested for the cytokine during previous studies (McLaren et al. 2010c; Miller et al. 2008). In order to study ADAMTS-4 promoter activity a luciferase gene reporter assay was implemented, where a deletion construct called p-789-LUC was transfected into RAW264.7 cells. The promoter construct spanned the -383 to +406 region of the ADAMTS-4 promoter (Mizui et al. 2000; Salter et al. 2011).

The data showed, for the first time, that ADAMTS-4 promoter activity (p-789-LUC) was suppressed by IL-33 stimulation (Figure 5.10). Of the ADAMTS family of proteases, only ADAMTS-4 and -5 promoter regions have been characterised (Mizui et al. 2000; Thirunavukkarasu et al. 2006; Thirunavukkarasu et al. 2007). The ADAMTS-4 promoter was shown to contain consensus-binding sites for many transcription factors including nuclear factor of activated T-cells (NF-AT), NF-1, specificity protein-1 (Sp1), activating

protein-2 (AP-2), runt-related transcription factor (Runx) and PEA3 (Mizui et al. 2000; Thirunavukkarasu et al. 2006). In the study by Mizui and colleagues (2000), it was suggested that the -383 to +10 region of the ADAMTS-4 promoter was required for full promoter activity. This region of the promoter was shown to contain one site for Sp1 and three AP-2 sites (Mizui et al. 2000). When the activities of various deletion constructs were investigated it was suggested that silencer elements were present between -726 and -384 (Mizui et al. 2000). The identification of a NF-1 binding site in this region could explain the silencing effect that was observed, as NF-1 transcription factors have been previously suggested to negatively regulate a number of genes (Mizui et al. 2000; Nakamura et al. 2001). Interestingly, the p-789-LUC deletion construct that was used during this investigation did not include this region that has been suggested to have silencing properties (-726 to -384). This suggests that the reduction in ADAMTS-4 promoter activity by IL-33 was produced independently of this NF-1 transcription factor-binding site. The silencing effect that was observed during this study could have been mediated through another NF-1 transcription factor-binding site that is present within the deletion construct (Figure 5.8).

An interesting future perspective to investigate the regulation of ADAMTS gene transcription would be to use various deletion constructs with different sizes within this experimental system. This would allow the minimal response region of the promoter that was responsible for the negative regulation of ADAMTS-4 to be identified. Following on from this, chromatin immunoprecipitation (ChIP) analysis could be carried out to identify potential DNA:protein interactions therefore beginning to pinpoint specific transcription factors that could be involved in reducing ADAMTS-4 promoter activity and where they bind to bring about their regulation. The precise roles could be confirmed by mutating specific promoter sites and analysing their ability to confer the response, to a heterologous promoter.

In the long term, it would be of interest to create luciferase gene reporter constructs for ADAMTS-1 and -5 so that similar experiments can be performed for these promoters. This will allow some comparisons between how the different ADAMTS family member promoters function to control gene transcription.

5.4.2 – IL-33 binding to the ST2 receptor causes down regulation of ADAMTS-1 and -4 expressions

It was of interest to determine if the ST2 receptor was involved in the transduction of the cytokine-mediated regulation of ADAMTS-1 and -4 gene expressions. In previous studies the ST2 receptor has been widely shown to be the physiological receptor for IL-33 (Kakkar and Lee 2008).

BMDMs from $ST2^{-/-}$ mice and WT mice were stimulated with 10ng/ml mIL-33 for 24hrs. The expression of ADAMTS-1 and -4 mRNAs were analysed using Q-PCR. It was shown that when the WT mice BMDMs were stimulated with mIL-33, the expression of ADAMTS-1 and -4 was reduced (Figure 5.11). When the stimulation was repeated on $ST2^{-/-}$ BMDMs the reduction in ADAMTS-1 and -4 expressions were lost (Figure 5.11). These data suggest that the binding of IL-33 to the ST2 receptor is critical to the regulation of ADAMTS-1 and -4 expressions in macrophages. This finding is consistent with many other studies that have shown that the ST2 receptor is involved in IL-33 gene regulation (Kakkar and Lee 2008; Miller and Liew 2011). IL-33 has been shown to promote inflammation by inducing lymphocytic infiltration in a ST2 independent manner, *in vivo* (Luzina et al. 2012). However, the majority of studies in relation to the regulation of gene transcription have suggested a ST2 dependent mechanism of transduction (Luzina et al. 2012).

An interesting future perspective would be to investigate which accessory proteins are required for the IL-33 response to be transduced through the ST2 receptor to reduce ADAMTS-1 and -4 expressions. For example, IL-1RAcP has been shown to be critical for IL-33 responses (Ali et al. 2007; Palmer et al. 2008), is the IL-33 regulation over ADAMTS-1 and -4 transduced via this mechanism? Other co-factors could influence the transduction of the IL-33 response such as MyD88, TRAF6, IRAK-1, IRAK-4 and JAK-2 (Funakoshi-Tago et al. 2008; Funakoshi-Tago et al. 2011; Miller and Liew 2011). It would also be of interest to study if the IL-33 response is transduced through these pathways. The obvious experimental strategy that could be employed to study these pathways would be RNAi,

followed by Q-PCR analysis. Also the use of BMDMs from knockout/conditional knockout mice could be utilised to perform these experiments.

5.4.3 – IL-33 causes activation of multiple signalling cascades

Some previously published papers have already investigated and presented which signalling pathways were activated after IL-33 stimulation of the ST2 receptor under various conditions (reviewed in Table 5.1). The results that have been presented were obtained from multiple different cell types. Some of the responses that were observed in the previous studies were cell-type-specific. For example, one group showed activation of p38 in lung endothelial cells and no activation of p38 in lung epithelial cells (Yagami et al. 2010). Also ERK was activated in a large number of studies by IL-33, but not in the basophil-like chronic myelogenous leukemia cell line, KU812 (Tare et al. 2010). One study has previously shown which signalling pathways were activated by IL-33 in mouse peritoneal macrophages (Funakoshi-Tago et al. 2011). They found that ERK, JNK, p38 and NFκB were activated by IL-33 stimulation (Funakoshi-Tago et al. 2011).

It was of interest to investigate which signalling cascades were activated, particularly in human macrophages, after stimulation with IL-33 (10ng/ml) for 24hrs. Phosphorylation western blots and transfection-based assays were employed to show, for the first time, which signalling pathways were activated after IL-33 stimulation of THP-1 cells. The experiments provided a novel insight into how IL-33 regulation was transduced within human macrophages, suggesting that ERK and c-Jun are activated by IL-33 stimulation. The data also suggested that NFκB, Akt and p38α were not activated by IL-33 stimulation of macrophages.

The data obtained is not totally consistent with what has been presented previously (Table 5.1). A study carried out on mouse peritoneal macrophages suggested that IL-33 activated ERK, JNK, p38 and NFκB whereas; our data suggests p38 and NFκB were not activated in differentiated THP-1 cells (Funakoshi-Tago et al. 2011). An explanation for the differences in the two data sets could be that the current study was carried out on human tissue and the other study employed mouse peritoneal macrophages (Funakoshi-

Tago et al. 2011). This difference could be species-specific. Species-specific responses have been observed previously during the regulation of macrophage functions by the PPAR and LXR families of transcription factors (Rigamonti et al. 2008).

Also the concentration of IL-33 used in the previous study was 100ng/ml, which was 10 fold higher than the dose used within this chapter (Funakoshi-Tago et al. 2011). Another difference is that the length of IL-33 stimulation varied between experiments. In the current study we stimulated for 24hrs, in previous studies much shorter stimulations had been used. The stimulations ranged from 5min to 2hrs in length (Funakoshi-Tago et al. 2008; Kamekura et al. 2012; Tare et al. 2010; Yagami et al. 2010).

Some possible future experiments could be performed that investigate the activation of signalling pathways using SDS-PAGE and western blots over a wide range of IL-33 stimulation times (5mins-24hrs). This would allow a greater understanding of how and when the regulation is transduced within macrophages. Some interesting future experiments could be planned to try and further investigate if NFκB activation occurs after IL-33 stimulation of macrophages. This is because only one experiment was carried out during this current study (Figure 5.16). Investigating the phosphorylation state of IκB by SDS-PAGE and western blot analysis, or following the time-dependent degradation of IκB could achieve these aims. Upon activation of the NFκB pathway, IκB becomes phosphorylated allowing it to dissociate from the NFκB dimer and undergo subsequent degradation (Nogueira et al. 2011).

5.4.4 – RNAi and IL-33 signal transduction

Following on from the activation studies, RNAi was used to investigate which signalling molecules appear to be involved in transducing the IL-33-mediated reduction in ADAMTS-1 and -4 expressions. The data obtained from this investigation are presented as a summary, in Table 5.5. The pattern of signal transduction was also conserved between THP-1 cells and HMDMs.

Table 5.5: Involvement of the MAPKs and PI3Ks in IL-33 regulated expression of ADAMTS-1 and -4 from THP-1 macrophages and HMDMs

Gene	ERK-1	ERK-2	p38	PI3Kγ	PI3Kδ	JNK-1/2	c-Jun	NFκB
ADAMTS-1	Y	Y	N	Y	Y	Y	Y	?
ADAMTS-4	Y	Y	N	Y	Y	Y	Y	N

Y = Involved, N = Not Involved and ? = Unknown

The data obtained from these experiments provide a novel insight into how IL-33 regulates ADAMTS-1 and -4 expressions from macrophages. ERK-1, ERK-2, PI3Kγ, PI3Kδ, JNK and c-Jun were involved in transducing the reduction of ADAMTS-1 and -4 expressions by IL-33, but p38 and NFκB were not involved (Table 5.5). During Section 5.3.3 it was shown that ERK-1 and -2 were activated after IL-33 stimulation of differentiated THP-1 cells (Figure 5.12). These data when combined with data from Table 5.5 suggest an ERK dependent mechanism of IL-33 signal transduction to regulate ADAMTS-1 and -4 expressions. During Section 5.3.3 it was also shown that c-Jun was activated by IL-33 stimulation of differentiated THP-1 cells (Figure 5.14). These data suggest a JNK and c-Jun dependent mechanism of IL-33 signal transduction to regulate ADAMTS-1 and -4 expressions. During Section 5.3.3 it was shown that Akt was not activated after IL-33 stimulation of differentiated THP-1 cells (Figure 5.15). These data when combined with data from Table 5.5 suggest a PI3K dependent mechanism of signal transduction. However, the response is not transduced through Akt and therefore could be mediated through PDK1 or PKC (Heinonen et al. 2008). It would be interesting to try and elucidate the activation of these components of the PI3K signalling pathway to elucidate if they are activated by IL-33 stimulation of human macrophages.

The array of different cell types that have been used to study how IL-33 signals makes it very difficult to make any direct comparisons between the current obtained data and previous findings. The novel observations that are presented within this chapter were obtained using RNAi as an inhibitory tool for silencing macrophage signalling pathways. All of the other studies that have investigated IL-33 signalling have used pharmacological inhibitors of the various signalling cascades (Table 5.1). There are advantages and disadvantages which come with the two described silencing techniques (Eggert et al.

2006). Pharmacological inhibitors are usually the first method of investigation when beginning a series of experiments. However, the findings need to be confirmed with knockdown/knockout approaches due to the associated non-specific actions of chemical inhibitors (Cohen 2010). The use of RNAi enables very specific knockdown of selected isoforms of proteins with limited off-target effects (Eggert et al. 2006). The previous studies also use different concentrations of IL-33 ranging from 20ng/ml to 100ng/ml in their experiments and they investigated regulation of responses from different genes including IL-8, IL-13 and IL-4 (Funakoshi-Tago et al. 2008; Kamekura et al. 2012; Tare et al. 2010; Yagami et al. 2010). It would be of interest to try and standardise the concentration and length of stimulation with IL-33 in future studies, as this would allow direct comparisons to be made between different studies. However, gene-specific and cell-type-specific responses will always be present.

When the RNAi experiments were being carried out it was of importance to investigate how the constitutive expressions of ADAMTS-1 and -4 from differentiated THP-1 cells were regulated. In a previous study the constitutive expression of ADAMTS-4 in THP-1 cells, was regulated by p38 activity (Salter et al. 2011). The constitutive expression of ADAMTS-4 in THP-1 differentiated with PMA has also been shown to be controlled by PPARγ and RXR activity (Worley et al. 2003). In the current study, the data suggests that the constitutive expressions of ADAMTS-1 and -4 were dependent on p38 activity. The expression of ADAMTS-1 was also dependent on PI3Kγ (Table 5.3). However, the constitutive expression of ADAMTS-1 was not dependent on PI3Kγ in HMDMs (Table 5.4); this difference could be PMA-rather than differentiation specific.

These data combined with the previous studies suggest that multiple signalling pathways are responsible for controlling the constitutive expression of ADAMTS proteases in macrophages. It is important that these pathways are thoroughly characterised, allowing a greater understanding of how the expression and activities are regulated within atherosclerosis. The data produced in this study allows comparison with the previous studies that were carried out. This is because all of the studies were performed using the THP-1 cell model that was differentiated with PMA for 24hrs (Salter et al. 2011; Worley et al. 2003).

In future studies relating to these experiments, it would be interesting to study if NFκB is involved in transducing the IL-33-mediated reduction in ADAMTS-1 and -5 expressions; this would involve the production of an ADAMTS-1 and -5 gene reporter construct. Other experimental options would be to use pharmacological inhibitors of NFκB activity, shRNA vectors targeting NFκB and cells from NFκB knockout mice. The regulation of ADAMTS-1 and -4 expressions by IL-33 could then be analysed within these experimental systems by QPCR. Selective pharmalogical inhibitors that target the MAPK signalling pathways could also be employed. This would provide independent confirmation for the data that was produced using RNAi. One last option would be to use BMDMs from knockout mice. It is possible to create p38, ERK-1, JNK-1/2, PI3Kγ and PI3Kδ knockout mice that are viable and physiologically normal (Bliss et al. 2009; Turban et al. 2005; Wang et al. 2006). However, ERK-2 and c-Jun knockout mice are not viable so macrophage/myeloid specific conditional knockout mice would be required (Bliss et al. 2009; Young et al. 1999).

It would also be of interest to try and pinpoint which transcription factors were activated by each of the pathways that were involved in the transduction of the response. The selection of activated transcription factors would help to understand how the activation of the signalling pathways lead to a reduction in gene transcription and ultimately a reduction in ADAMTS-1 and -4 gene expression.

5.4.5 – Future work within this thesis

In the current chapter the mechanism by which IL-33 regulates ADAMTS-4 and -5 expressions have been delineated. The next chapter presented within this thesis will investigate the roles that ADAMTS proteases have in the plaque during disease progression after they have been expressed. The cellular roles of the ADAMTS proteases during atherosclerosis are poorly understood and require more research.

Chapter 6

Designing adenoviral vectors to deliver shRNAs targeting ADAMTS-1, -4 and -5

6.1 – Introduction

6.1.1 – Re-engineering adenoviral vector systems for analyses of gene function

RNAi is useful tool during the investigation of gene function in cells (Meister and Tuschl 2004). An understanding of the biological role of any gene comes only after observing the phenotypic consequences of altering the function of that gene in a living cell or organism (Paddison et al. 2002). The concept and methods of RNAi were introduced in Chapter 4, where siRNA and shRNA were used to silence the activity of specific signalling cascades in human macrophages after IL-33 stimulation. The work presented in this chapter involves designing and making an adenoviral vector to deliver shRNA targeting ADAMTS-1, -4 and -5. The direct insertion method used to engineer the adenoviral vectors was developed by Gavin W. G. Wilkinson's laboratory in Cardiff University (Stanton et al. 2008).

The adenovirus with zero cloning steps (AdZ) system was designed to allow cloning of a shRNA directly into a replication deficient recombinant adenovirus vector (AdZ-5 vector) carried on a bacterial artificial chromosome (BAC) (Stanton et al. 2008). The AdZ vectors contain the Ad5 vector genome, deleted for E1 and E3 regions in a single copy vector (Stanton et al. 2008). It also contains the HCMV major intermediate early promoter and polyA signal sequence, with a cassette coding for ampicillin resistance, *LacZα* and *SacB* (Stanton et al. 2008). SacB encodes for a gene giving sensitivity to sucrose and *LacZα* provides blue/white screening after growth with X-gal. The shRNA cloning takes place in a single, simple, recombineering step within SW102 *E.coli* cells carrying the AdZ BAC

(Stanton et al. 2008). There is no conventional cloning step; the gene is simply recombineered directionally into the vector by lambda red proteins, and selectable markers permit the easy identification of positive colonies. The AdZ vector system is robust and straightforward and it is suited to occasional use or high-throughput applications. The AdZ vector is displayed in Figure 6.1.

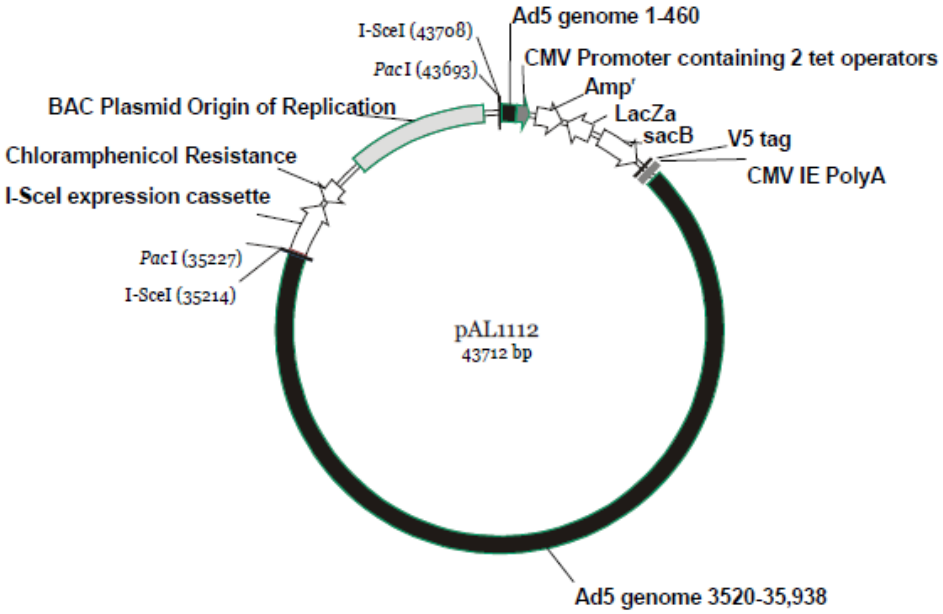


Figure 6.1: **A diagram of the complete AdZ plasmid**

A scale diagram of pAL1112 showing features common to all AdZ vectors, including the self-excision system (I-SceI), tet-restricted CMV promoter and polyadenylation sequence with the sacB/LacZ selectable marker and epitope tag (Stanton et al. 2008).

The vector that was chosen for use within this chapter allowed the insertion of shRNAs by recombination of synthesised oligonucleotides (Stanton et al. 2008). pAdZ-miR155 vectors have miR-155 flanking arms of homology on either side of the Amp/*SacB*/*LacZ* selectable marker (Chung et al. 2006). When recombination of the oligonucleotides is successful the cassette containing Amp/*SacB*/*LacZ* will be replaced by the shRNA containing oligonucleotides (Stanton et al. 2008). This allows for selection of positive colonies by growing the bacteria on plates containing 5% sucrose and X-gal, the resulting outcomes of the positive and negative colonies are outlined in Figure 6.2.

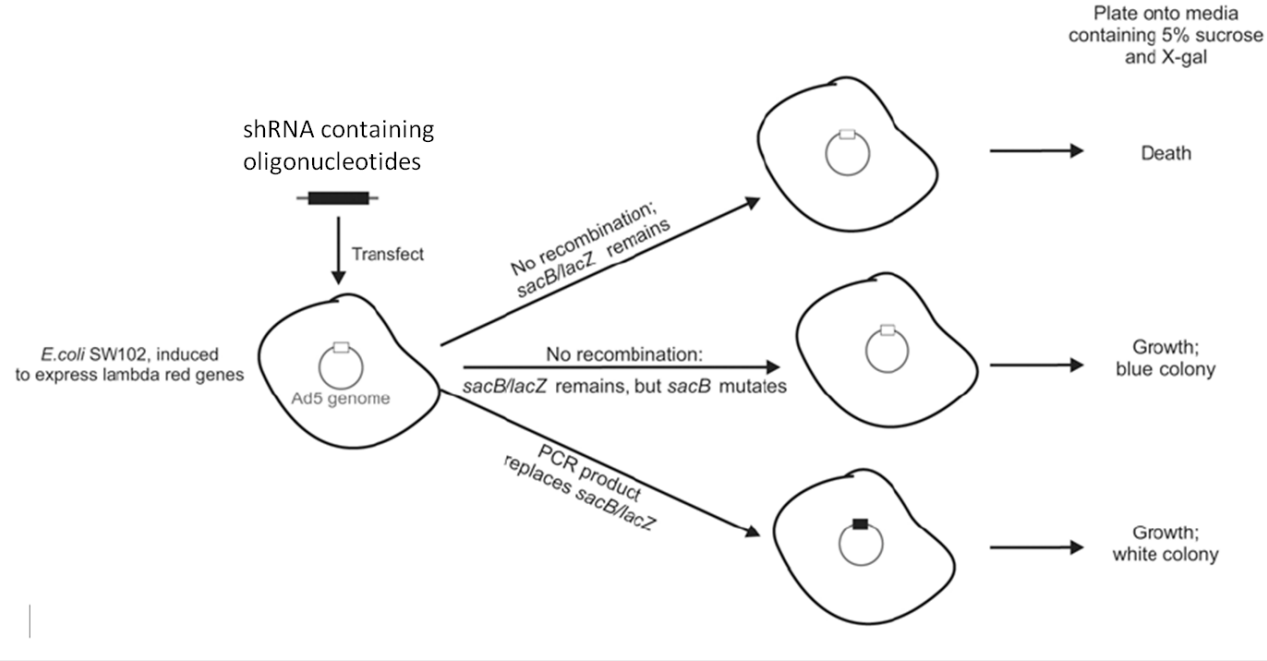


Figure 6.2: **Identification of positive colonies during recombination**

The schematic diagram displays the possible outcomes after recombination. The selection of positive colonies is achieved by selecting with 5% sucrose and X-gal (Stanton et al. 2008).

Following confirmation that the correct recombination has occurred by restriction digestion and sequencing, the vector DNA is transfected into packaging cells in order to grow the recombinant adenovirus (Stanton et al. 2008). The vectors are not replication competent as the E1 region of the AdZ genome has been removed. Cell lines that express E1 functions, such as 293TREx (Invitrogen), are therefore essential for adenoviral propagation (Stanton et al. 2008).

The resulting viral particles are harvested from the cells and the numbers of viral particles are determined by accelerated viral titering (Bewig and Schmidt 2000).

6.1.3 – ADAMTS cellular roles

RNAi has successfully been used in previous studies to try and understand the cellular roles of ADAMTS proteases in various cell types and disease conditions (Salter et al. 2010;

Tortorella et al. 2009; Wang et al. 2009). Some examples of the methods used to carry out RNAi along with the findings will be reviewed in the following section.

One of the major disease conditions that ADAMTS proteases have been implicated in is OA (Huang and Wu 2008). ADAMTS proteases have been shown to have a major role in aggrecan breakdown of articular cartilage (Huang and Wu 2008). The understanding of the role that ADAMTS proteases play in OA has come from RNAi experiments *in vitro,* followed by gene knockdown experiments *in vivo*. One experiment that was carried out, utilised siRNA that targeted ADAMTS-1, -4 and -5 in human cartilage explant cultures (Song et al. 2007). The knockdown of ADAMTS-4 and -5 attenuated aggrecan degradation in the explant cultures and showed that ADAMTS-1 was not involved during aggrecan breakdown (Song et al. 2007). Following on from this experiment, siRNA targeting ADAMTS-7 and -12 was utilised to assess their role in COMP degradation during OA (Luan et al. 2008). During *in vitro* digestion assays, knockdown of ADAMTS-7 and -12 attenuated the IL-1β and TNF-α induced COMP degradation of cartilage explants from OA patients (Luan et al. 2008). Also, lentiviral delivery of ADAMTS-5 and -9 shRNA resulted in an increased matrix deposition in human 3D chondrocyte cultures (Coughlan et al. 2010).

One study also utilised siRNA to investigate the function of ADAMTS-7 during VSMC migration after balloon injury of carotid arteries (Wang et al. 2009). This study highlights the importance of using RNAi to assess gene function in different disease states. The previously major characterised role for ADAMTS proteases was involvement in OA and the involvement in atherosclerosis was not so well understood (Salter et al. 2010). The study showed that after balloon injury of rat carotid arteries ADAMTS-7 expression was induced (Wang et al. 2009). In primary rat VSMCs, ADAMTS-7 over-expression exacerbated VSMC migration and ADAMTS-7 siRNA treatment attenuated VSMC migration, *in vitro* (Wang et al. 2009). The functions of ADAMTS proteases during atherosclerosis progression need further research, as our current understanding is relatively poor (Salter et al. 2010).

6.2 – Experimental Aims

The primary experimental aim of this experimental chapter was to design and produce an adenoviral vector to deliver shRNAs that target ADAMTS-1, -4 and -5. Once the adenoviral vectors had been produced and their responses verified, they were planned to be used in assays to try and assess the functional roles that ADAMTS-1, -4 and -5 play during atherosclerosis.

Firstly, an in vitro Boyden chamber migration assay was designed and used to investigate if ADAMTS-1, -4 or -5 expressions were involved in the regulation of macrophage migration.

Secondly, Amplex Red cholesterol assays were utilised to investigate if ADAMTS-1, -4 or -5 expressions regulate the uptake of AcLDL by human macrophages during atherosclerosis.

The strategies that were employed during this experimental chapter are outlined in Figures 6.3 – 6.5.

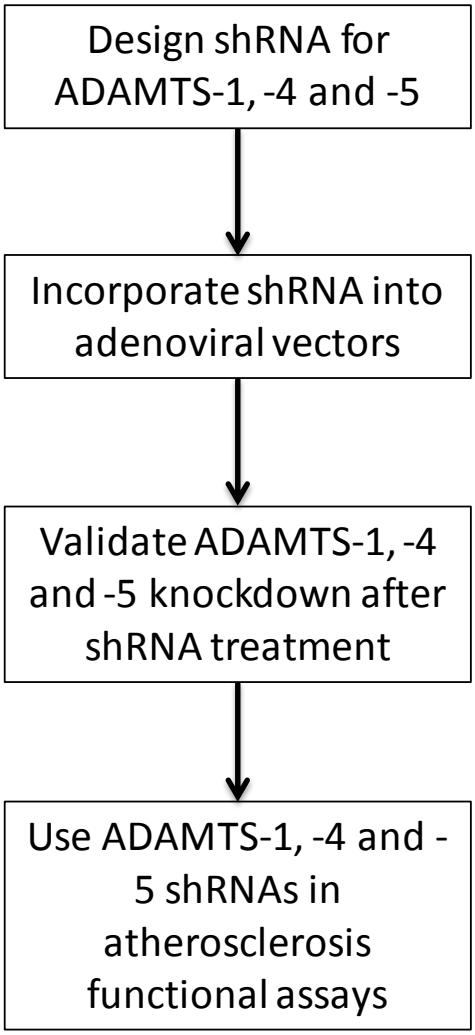


Figure 6.3: **Designing and making ADAMTS-1, -4 and -5 shRNA experimental strategy**

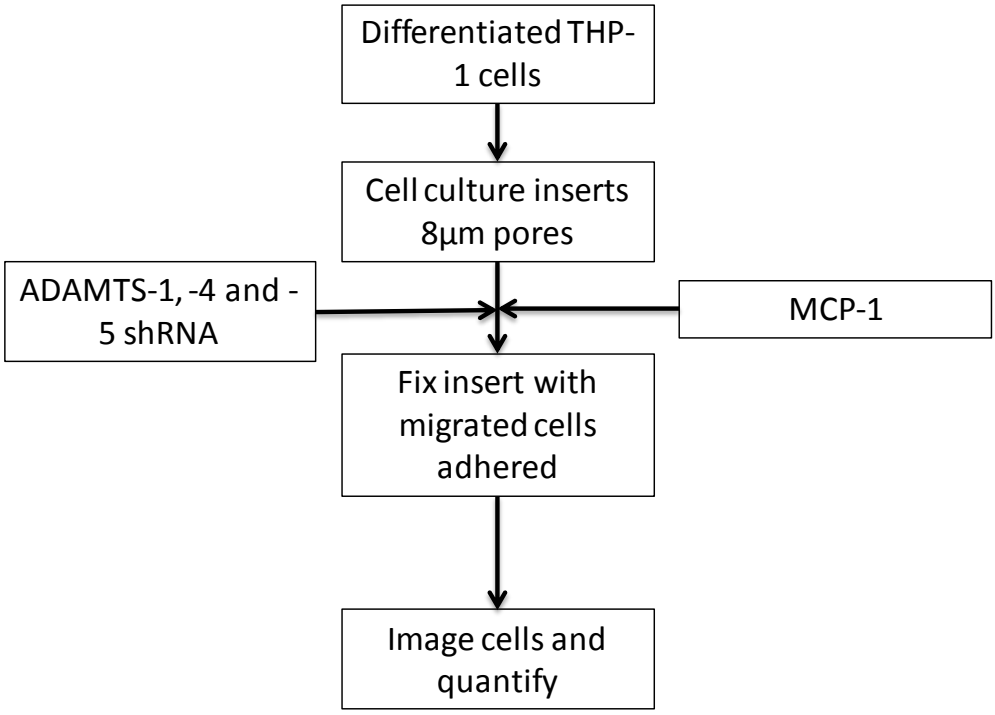


Figure 6.4: **Boyden chamber migration assay experimental strategy**

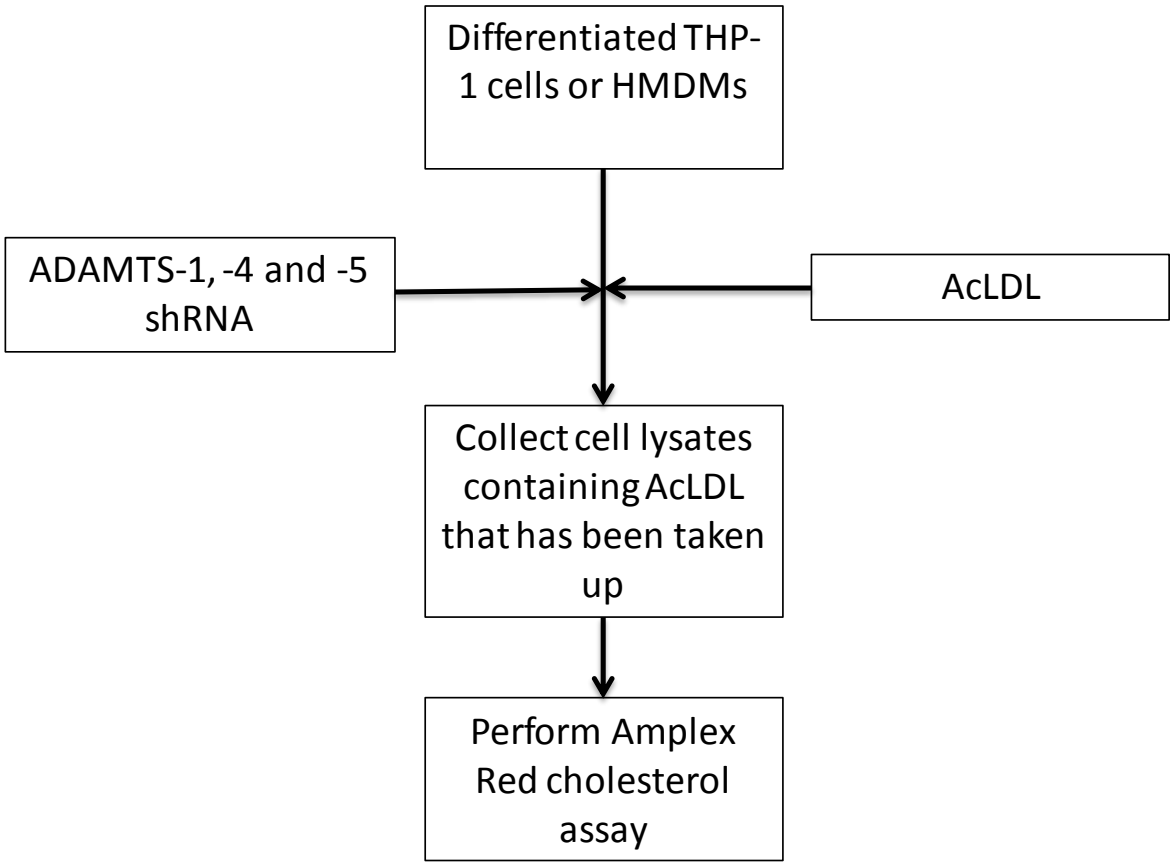


Figure 6.5: **Amplex Red cholesterol assay experimental strategy**

6.3 – Results

6.3.1 – Designing and making adenoviral vectors to deliver shRNA targeting ADAMTS-1, -4 and -5

When the shRNA adenoviral vectors targeting ADAMTS-1, -4 and -5 were made, originally multiple target sequences were designed for each gene. Multiple target sequences were designed in order to improve the probability of successfully producing a functional shRNA delivery vector. The different target sequences were all processed through the whole protocol, producing multiple adenoviral vectors that could target either ADAMTS-1, -4 or -5.

The efficiency of knockdown was analysed by QPCR and the viruses with the highest titre and/or most potent knockdown effect over ADAMTS-1, -4 and -5 were selected for all further experiments.

The design of the shRNA sequence was performed using an algorithm on the Invitrogen website. The algorithm was called RNAi Designer (Invitrogen, UK); the suggested target sequences produced for ADAMTS-1, -4 and -5 are displayed in Table 6.1.

Table 6.1: **Sequences of the RNA to be targeted by shRNA**

shRNA target gene	Gene accession number	shRNA target sequence (5'-3')	Radz number
ADAMTS-1	NM_006988	ACTTGTGTGGGTCCCAGACAT GCAAACGAGTGCGCTACAGAT GCTTCCGAGTGTGCAAAGGAA	Radz029 Radz030 Radz031
ADAMTS-4	NM_005099	TCAGCCTTCACTGCTGCTCAT AGGCACTGGGCTACTACTATG	Radz027 Radz028
ADAMTS-5	NM_007038	TTTGCCTATCGTCACTGTAAT CTAGATTCACTGCCTATTTAG	Radz032 Radz033

For the recombination, forward and reverse oligonucleotides were designed for each target sequence that contained complementary coding regions for the classical stem-loop shRNA precursor, with flanking homology to pAdZ-miR155 plasmid (Chung et al. 2006). The structure of the stem-loop precursor was designed to mimic the physiological miR155 as much as possible, to this end the length of the shRNA coding strand was designed to be 2 bases longer than complementary non-coding strand, to maximize shRNA processing and strand selectivity (Chung et al. 2006). An example of the oligonucleotides that were used for the recombination of Radz031, 027 and 033 are displayed in Table 6.2. The same principle was used to design all of the other oligonucleotides.

Table 6.2: **Examples of oligonucleotides sequences (Radz031, 027 and 033)**

Oligonucleotide	Sequence (5'-3')
ADAMTS-1 (031) forward oligo	**CAGCCTGGATCCCTGGAGGCTTGCTGAAGGCTGTATGCTG**TTCCTTTGCACA CTCGGAAGCGTTTTGGCCACTGACTGACGCT
ADAMTS-1 (031) reverse oligo	**TTTGTTCCATGTGAGTGCTAGTAACAGGCCTTGTGTCCTG**TTCCTTTGCACTC GGAAGCGTCAGTCAGTGGCCAAAACGCT
ADAMTS-4 (027) forward oligo	**CAGCCTGGATCCCTGGAGGCTTGCTGAAGGCTGTATGCTG**ATGAGCAGCAG TGAAGGCTGAGTTTTGGCCACTGACTGACTCA
ADAMTS-4 (027) reverse oligo	**TTTGTTCCATGTGAGTGCTAGTAACAGGCCTTGTGTCCTG**ATGAGCAGCAGA AGGCTGAGTCAGTCAGTGGCCAAAACTCA
ADAMTS-5 (033) forward oligo	**CAGCCTGGATCCCTGGAGGCTTGCTGAAGGCTGTATGCTG**CTAAATAGGCA GTGAATCTAGGTTTTGGCCACTGACTGACCTA
ADAMTS-5 (033) reverse oligo	**TTTGTTCCATGTGAGTGCTAGTAACAGGCCTTGTGTCCTG**CTAAATAGGCAG AATCTAGGTCAGTCAGTGGCCAAAACCTA

The oligonucleotides were designed to produce the classical stem-loop structure of shRNAs when it was transcribed. The forward oligonucleotide sequences consist of 40 bases of vector homology (black) to aid homologous recombination, 21 complimentary bases (antisense) to the shRNA target sequence (green), a 19 base loop sequence (blue) and 3 identical bases (sense) to the shRNA target sequence (red). The reverse oligonucleotide sequences consist of 40 bases of vector homology (black), 19 bases of sense to the target (red), 19 base loop (blue) and 3 bases of antisense to the target (green).

The mechanism of how the oligonucleotides produce the classical stem-loop structure of shRNA is outlined in Figure 6.6.

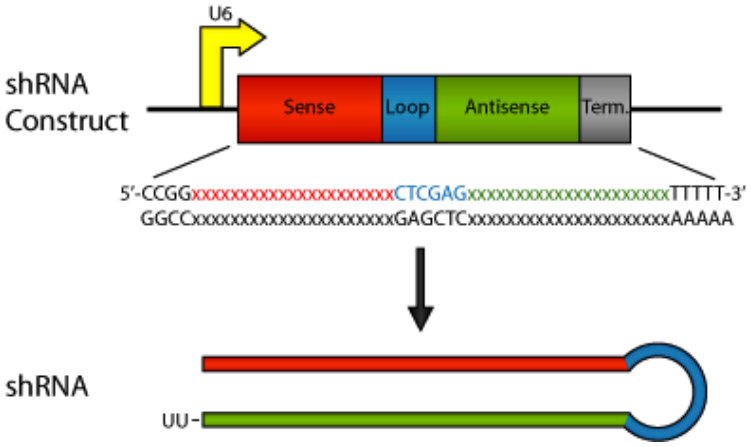


Figure 6.6: The transcription of the classical stem-loop structure of shRNAs (Image adapted from addgene)

A schematic of how a shRNA construct is transcribed to produce the classical stem-loop structure of the resulting shRNA. The two loops are either homologous (sense) or complimentary (antisense) to the original target shRNA sequence. They are linked by a loop sequence.

The lambda red recombination was carried out in SW102 cells already containing the pAdZ-miR155 plasmid; the oligonucleotides were introduced to the cells by electroporation. Following the recombination step, positive colonies were selected by plating the cells on media containing 5% sucrose and X-gal. Within positive colonies, the replacement of the Amp/*SacB*/*LacZ* cassette with the inserted oligonucleotides caused white colonies to grow on the selective media. One problem at this stage was the presence of some false-positive colonies that appear white, but still contain the Amp/*SacB*/*LacZ* cassette. The phenotypic appearances of the true-positive and the false-positive colonies were quite different; Figure 6.7 aided the identification of the true-positive colonies.

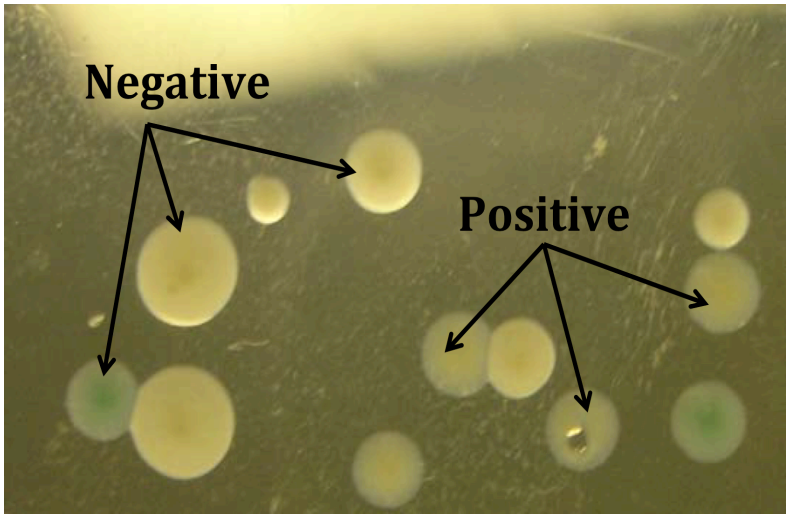


Figure 6.7: Selection of positive colonies containing the AdZ vector after recombination

One technical problem observed during the protocol was that false-positive white colonies were produced during the blue/white screening process. The reason for the occurrence of these false-positive colonies is unknown, but the phenotypic appearance was different to that of a true-positive colony. The false-positive appear to be more opaque. The above image was kindly provided by Richard Stanton (Cardiff University).

After the positive colonies had been selected and expanded, the resulting plasmid was purified. The success of the recombination was verified by carrying out a restriction digest of the plasmid, followed by DNA sequencing to confirm that the oligonucleotides had been incorporated into the AdZ plasmid. An example of the digest that was performed on a positive and negative colony is displayed in Figure 6.8.

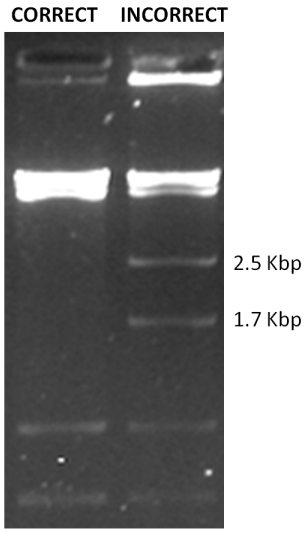


Figure 6.8: Restriction digests of a positive and negative colony

The AdZ plasmids give restriction bands of 18, 11, 7.7, 2.5, 1.7, 0.8 and 0.6 Kbp. The 2.5 and 1.7 Kbp bands are produced by restriction digestion of the Amp^-/SacB/LacZ cassette. In negative colonies these bands should be present. In positive colonies the Amp^- /SacB/LacZ cassette will be replaced by oligonucleotides, therefore the 2.5 and 1.7 Kbp bands will disappear. The oligonucleotides are very small in comparison and will not show on the gel.

Following confirmation that the recombination was successful, the plasmids were transfected into packaging cells to propagate the adenoviral particles. 293TREx cells (Invitrogen) were used for this purpose. After propagation the viral particles were extracted and titred using a technique called accelerated viral titering (Bewig and Schmidt 2000). The titering utilised the infection of 293TREx cells with varying dilutions of the viral stock. After infection, an antibody was applied that targeted the adenoviral particles within the 293TREx cells. After DAB staining, all infected cells were stained brown and a calculation was used to ascertain the titer of viral particles in the stock (Materials and Methods gives more details). An example of the DAB immune-staining is displayed in Figure 6.9.

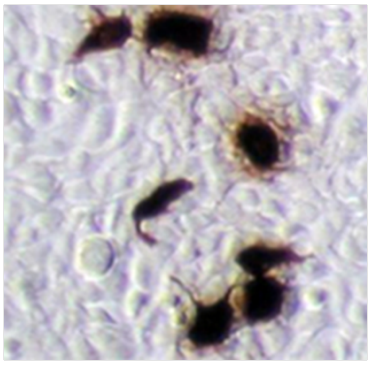


Figure 6.9: Adenoviral infection of a cell can be detected by DAB immune-staining
A monolayer of 293TREx cells was infected by adenoviral particles. An anti-adenoviral antibody was used to detect infected cells. After DAB staining infected cells were rendered brown. (The image was courtesy of Abcam.com).

6.3.2 – Infection of THP-1 cells with the adenoviral vector produced gene knockdown

After the titer of viral particles had been ascertained for each adenoviral vector, it was necessary to test the efficiency of the shRNA-mediated silencing of ADAMTS-1, -4 and -5 in human cells. The titres that were obtained for each different adenovirus are displayed in Table 6.3.

Table 6.3: Viral titres of the adenoviral vectors that were produced

Vector	Titre (pfu)
Radz029 (ADAMTS-1)	3.5×10^9
Radz030 (ADAMTS-1)	4.1×10^9
Radz031 (ADAMTS-1)	5.3×10^9
Radz027 (ADAMTS-4)	4.55×10^9
Radz028 (ADAMTS-4)	1.88×10^9
Radz032 (ADAMTS-5)	6.9×10^9
Radz033 (ADAMTS-5)	1.07×10^{10}

Firstly, the THP-1 cell line was chosen for the gene silencing of ADAMTS-1, -4 and -5 using the adenoviral shRNA vectors that had been produced. The cells were infected to a multiplicity of infection (MOI) of 100. The MOI had previously been optimised within our

laboratory using a GFP tagged adenoviral vector. In the previous experiment, >98% of the cells present were infected after using an MOI of 100 (data not shown). After the infection of differentiated THP-1 cells with the ADAMTS shRNAs, the expressions of ADAMTS-1, -4 and -5 mRNA were measured and compared to that of THP-1 cells that had been infected with a scrambled control shRNA (Figure 6.10). These data acted as an initial screen to test which adenoviruses had the most potent knockdown over ADAMTS-1, -4 and -5.

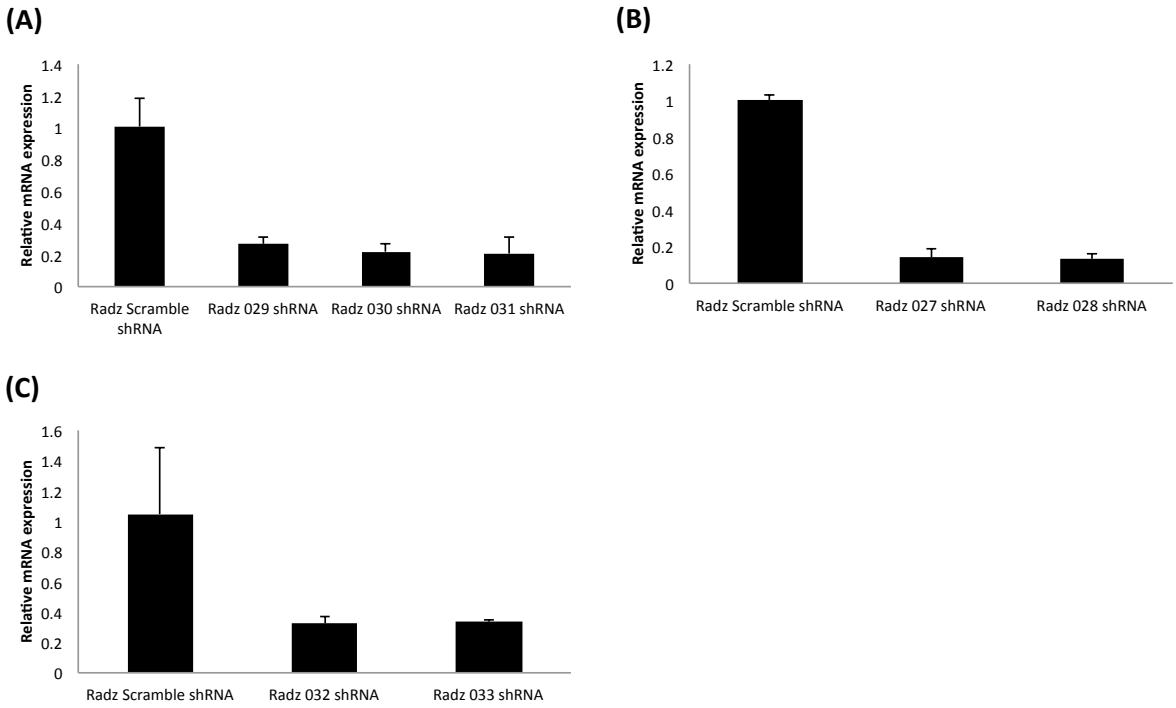


Figure 6.10: An initial screen of shRNAs targeting ADAMTS-1, -4 and -5 using differentiated THP-1 cells

Differentiated THP-1 cells were infected with adenoviruses expressing either a scramble control or ADAMTS-1, -4 or -5 shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (control values from Radz scramble samples were arbitrarily assigned as 1), from 1 experiment. The standard deviation within the triplicate samples are displayed on the graph.

It was observed that all of the adenoviral vectors produced were able to knockdown their target gene (Figure 6.10). Radz029, 030 and 031 all reduced the expression of ADAMTS-1 by around 74-80% (Figure 6.10, Panel A). Radz027 and 028 reduced the expression of ADAMTS-4 by around 86-87% (Figure 6.10, Panel B) and Radz032 and 033 reduce the expression of ADAMTS-5 by around 66-68% (Figure 6.10, Panel C). As all of the adenoviruses had a potent knockdown action, the choice was made to use the adenoviral vectors with the highest viral titre in all future experiments (Table 6.4).

Table 6.4: The adenoviral vectors targeting ADAMTS-1, -4 and -5 that were chosen to use in future experiments

Target	Adenovirus chosen
ADAMTS-1	Radz031
ADAMTS-4	Radz027
ADAMTS-5	Radz033

Once the viruses had been chosen, experiments were carried out that validated the knockdown of ADAMTS-1, -4 and -5 in differentiated THP-1 cells (Figure 6.11). The initial screen was only carried out once so no statistical analysis could be used, the validation experiments were repeated 3 times to enable statistical analysis.

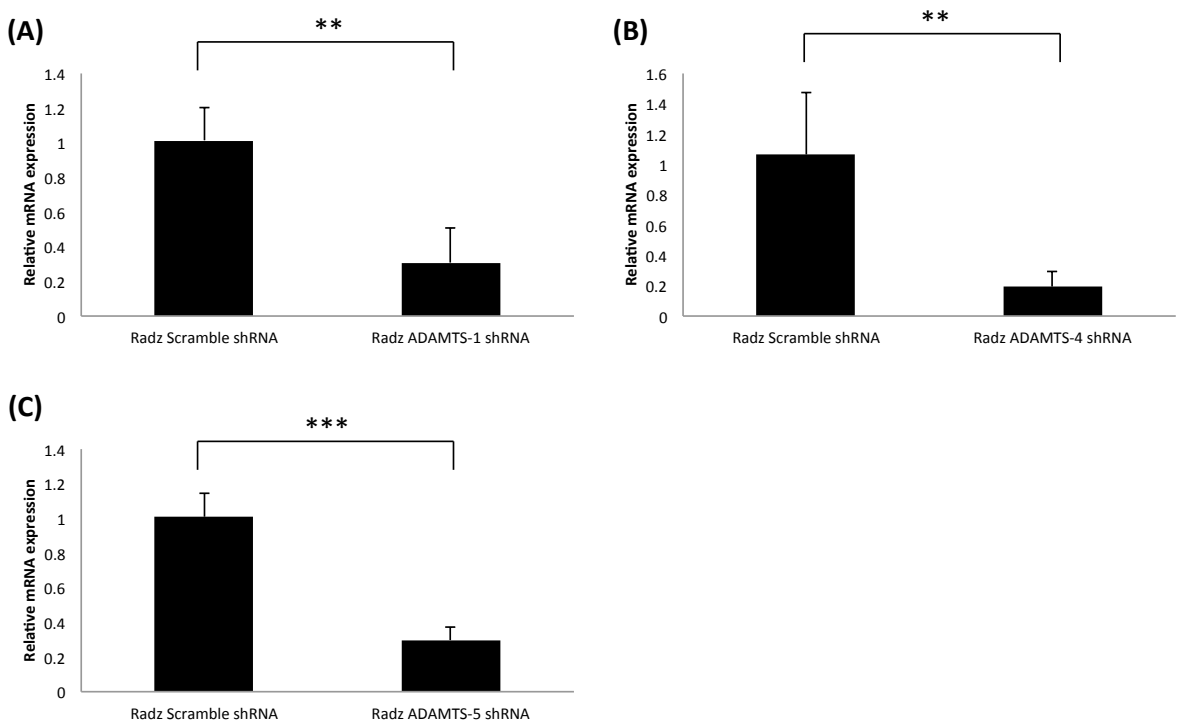


Figure 6.11: Infection of differentiated THP-1 cells with shRNA delivering vectors significantly reduced ADAMTS-1, -4 and -5 expressions

Differentiated THP-1 cells were infected with an adenovirus expressing either a scramble control or ADAMTS-1, -4 or -5 shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (control values from Radz scramble samples were arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. **P=<0.01, ***P=<0.001.

It was observed that all three adenoviral vectors targeting ADAMTS-1, -4 and -5 produced a significant reduction in mRNA expression in differentiated THP-1 cells (Figure 6.11). The ADAMTS-1 shRNA vector produced a 70% reduction, the ADAMTS-4 vector generated an 82% reduction and the ADAMTS-5 vector yielded a 71% reduction in mRNA expression. All of the viruses were used at a MOI of 100 as previously mentioned.

6.3.3 - Infection of HMDMs with the adenoviral vector produced gene knockdown

Following on from the experiments that were performed on differentiated THP-1 cells, it was of interest to confirm if the gene expression of ADAMTS-1, -4 and -5 could be reduced in HMDMs by the shRNA vectors (Figure 6.12).

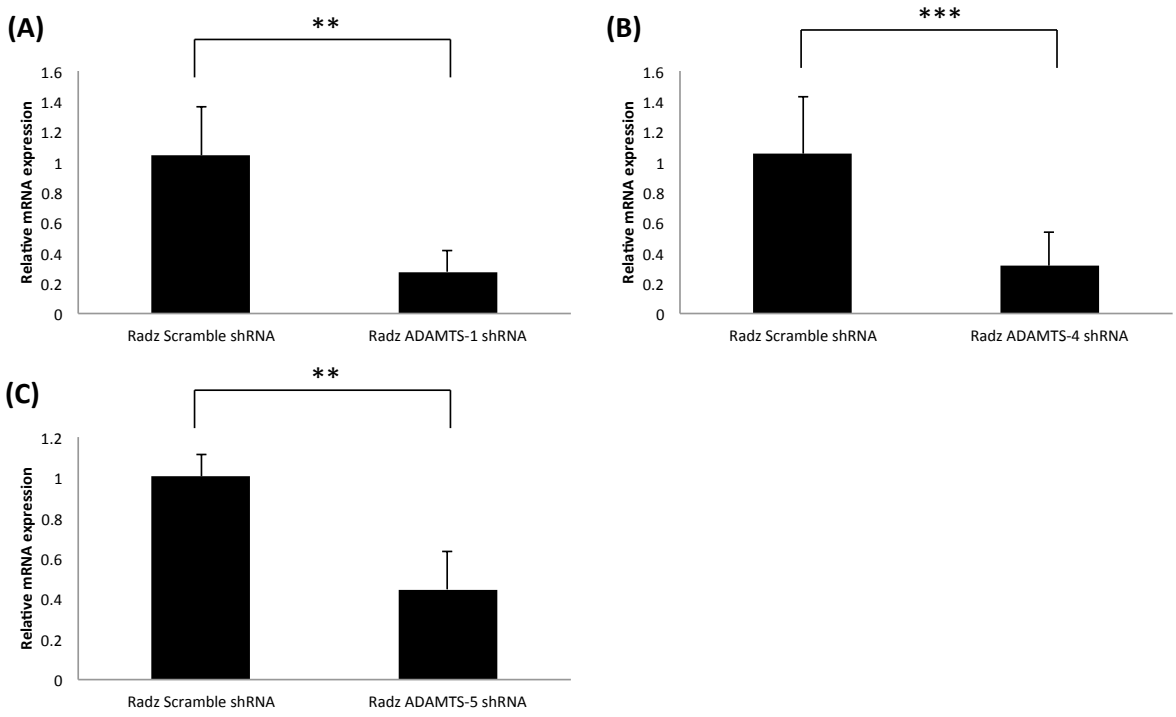


Figure 6.12: Infection of HMDMs with shRNA delivering vectors significantly reduced ADAMTS-1, -4 and -5 expressions

HMDMs were infected with an adenovirus expressing either a scramble control or ADAMTS-1, -4 or -5 shRNA. Total RNA samples were subjected to a reverse transcriptase reaction and Q-PCR analysis. Panel A = ADAMTS-1, B = ADAMTS-4 and C = ADAMTS-5. The graphs display the average fold change in mRNA expression in relation to the control (control values from Radz scramble samples were arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using the Student's t-test. **P=<0.01, ***P=<0.001.

It was observed that all three adenoviral vectors targeting ADAMTS-1, -4 and -5 produced a significant reduction in mRNA expression from HMDMs (Figure 6.12). The ADAMTS-1 shRNA vector produced a 73% reduction, the ADAMTS-4 vector generated a 69%

reduction and the ADAMTS-5 vector yielded a 54% reduction in mRNA expression. All of the viruses were used at a MOI of 100 to mimic the experiments that were carried out in differentiated THP-1 cells. When calculating the MOI for these experiments the cell number of the primary HMDMs was assumed to be 500,000 after differentiation had produced a complete monolayer.

6.3.4 – Migration assays and ADAMTS-1, -4 and -5 gene silencing

Once the adenoviral vectors that delivered shRNA for ADAMTS-1, -4 and -5 were fully validated in differentiated THP-1 cells and HMDMs, it was possible to include them as RNAi agents in functional cellular assays. The assays were designed to try and understand the functions that ADAMTS-1, -4 and -5 play within the disease, atherosclerosis.

Firstly, a modified Boyden chamber protocol was utilised to investigate if the activity of ADAMTS-1, -4 and -5 was involved in the regulation of macrophage migration. The mechanism of three-dimensional migration of macrophages during atherosclerosis is poorly understood and requires more research (Cougoule et al. 2010). Briefly, differentiated THP-1 cells were adhered to the upper chamber of the cell culture insert within the modified Boyden chamber setup. The cells were infected with either a scrambled shRNA or a shRNA targeting ADAMTS-1, -4 or -5. Following the use of RNAi, a chemotactic concentration gradient was created by the addition of 20ng/ml MCP-1 to the lower chamber. The cells that underwent migration were fixed, stained using DAPI and quantified using fluorescence microscopy and image analysis software (CellProfiler). The results from the assay are displayed in Figure 6.13).

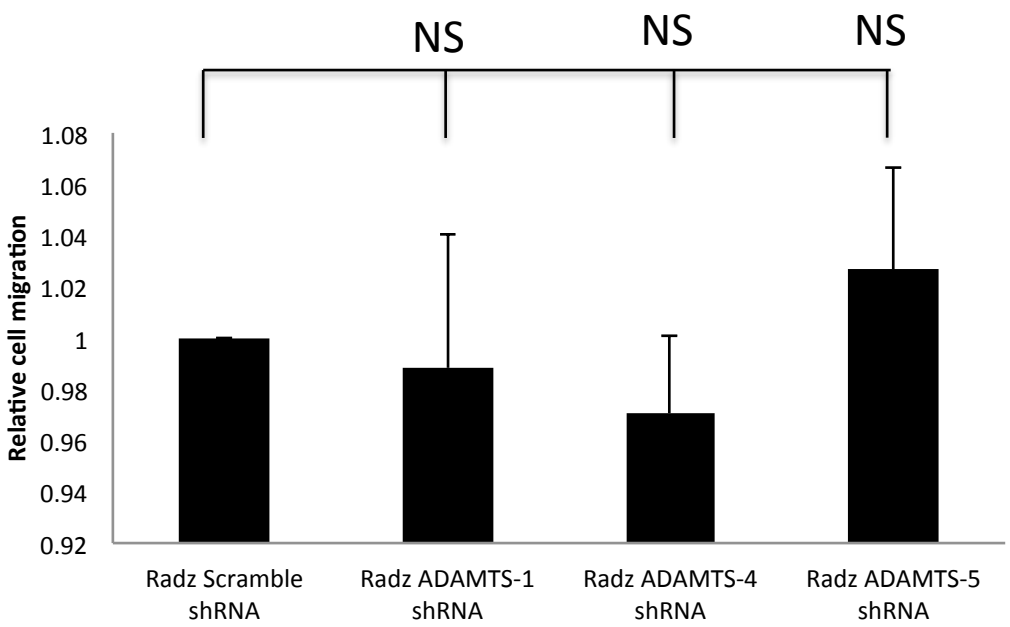


Figure 6.13: ADAMTS-1, -4 and -5 were not involved in the regulation of macrophage migration within an *in vitro* Boyden chamber assay

A modified Boyden chamber assay was performed using differentiated THP-1 cells. The cells were adhered to the top chamber and infected with either scrambled or ADAMTS-1, -4 or -5 shRNA. A chemotactic gradient was achieved by the addition of 20ng/ml MCP-1 to the lower chamber. The cells were left to migrate for 24hrs followed by the quantification of the migrated cells by fluorescent microscopy (DAPI) and image analysis. The graph displays the average fold change to migration in relation to the control (control values from Radz scramble samples were arbitrarily assigned as 1), from 3 independent experimental repeats. Statistical analysis was performing using one-way ANOVA.

It was observed that after RNAi had been carried out to target ADAMTS-1, -4 and -5, no significant change was observed to macrophage migration within the modified Boyden chamber assay (Figure 6.13).

6.3.5 – Amplex Red cholesterol uptake assay and ADAMTS-1, -4 and -5 gene silencing

Macrophage foam cell formation is a very important pathological process that occurs during the disease, atherosclerosis (McLaren et al. 2011). The regulation of foam cell formation by cytokines and other inflammatory mediators is well studied and understood

(McLaren et al. 2011). The role of the extracellular matrix and its cleavage by ADAMTS proteases during macrophage foam cell formation is understood to a lesser extent. A cellular assay that was able to quantify the amount of cholesterol being taken up by macrophages was utilised to investigate if ADAMTS-1, -4 and -5 regulated macrophage modified-LDL uptake. Differentiated THP-1 cells were used first during the investigations (Figure 6.14).

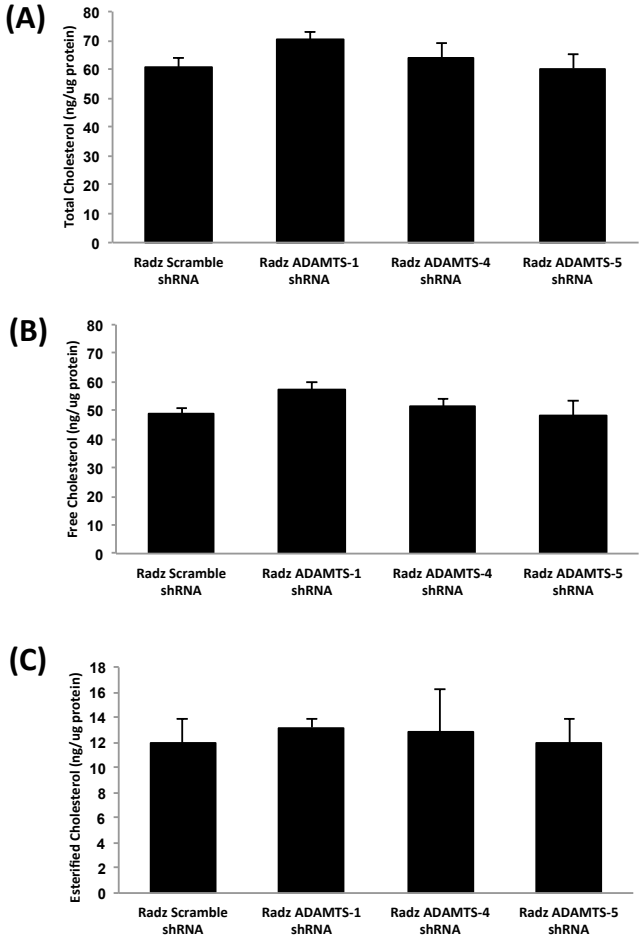


Figure 6.14: Uptake of Ac-LDL by differentiated THP-1 cells was not regulated by the activity of ADAMTS-1, -4 or -5

An Amplex Red cholesterol uptake assay was performed using differentiated THP-1 cells. The cells were infected with either scrambled, ADAMTS-1, -4 or -5 shRNA. The cells were incubated for 24hrs along with 10ng/ml Ac-LDL, followed by the quantification of total, free and esterified cholesterol within the cells. The graph displays the average amount of (A) total cholesterol, (B) free cholesterol and (C) esterified cholesterol from 1 experiment carried out in triplicate.

It was observed that after RNAi targeting ADAMTS-1, -4 and -5 there was no change in the amount of Ac-LDL that was taken up by differentiated THP-1 cells (Figure 6.14). The experiment was repeated in HMDM cells (Figure 6.15).

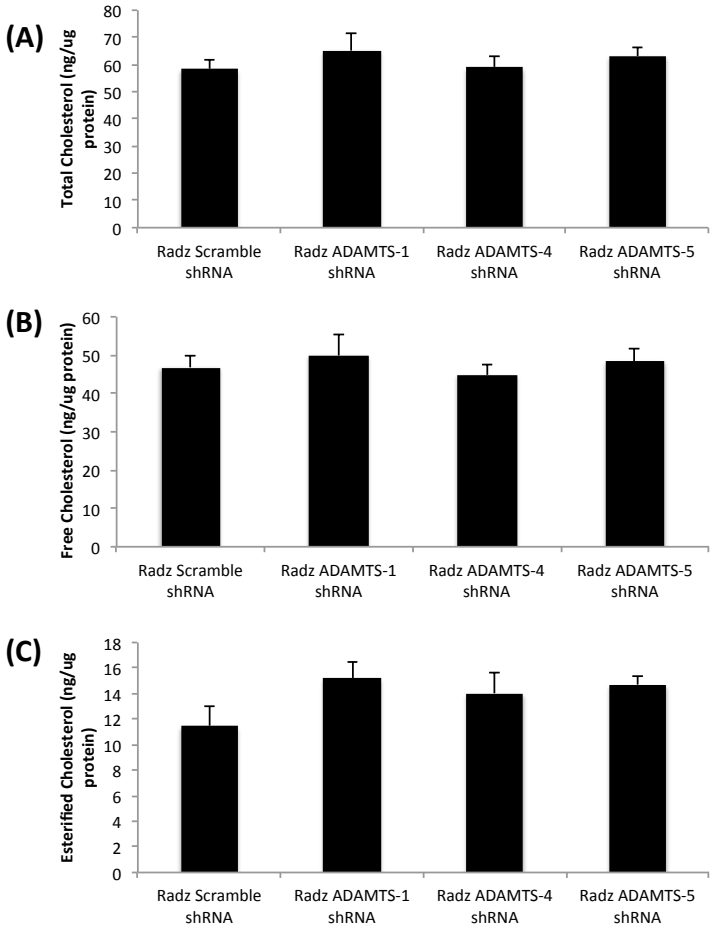


Figure 6.15: Uptake of Ac-LDL by HMDM cells was not regulated by the activity of ADAMTS-1, -4 or -5

An Amplex Red cholesterol uptake assay was performed using HMDM cells. The cells were infected with either scrambled, ADAMTS-1, -4 or -5 shRNA. The cells were incubated for 24hrs along with 10ng/ml Ac-LDL, followed by the quantification of total, free and esterified cholesterol within the cells. The graph displays the average amount of (A) total cholesterol, (B) free cholesterol and (C) esterified cholesterol from 2 independent experimental repeats carried out in triplicate.

It was observed that after RNAi targeting ADAMTS-1, -4 and -5 there was no change in the amount of Ac-LDL that was taken up by HMDM cells (Figure 6.15).

6.4 – Discussion

As mentioned in previous chapters of this thesis, the magnitude of research that it being undertaken to elucidate the role of the ADAMTS proteases during atherosclerosis is expanding (Salter et al. 2010). The expression and action of ADAMTS proteases has been investigated during atherosclerosis (Jonsson-Rylander et al. 2005; Salter et al. 2011; Salter et al. 2010; Wagsater et al. 2008). However, the actual cellular consequences of ADAMTS activity are poorly understood during the progression of atherosclerosis and this area requires further research (Salter et al. 2010).

Versican is an abundant proteoglycan within the walls of blood vessels (Wight and Merrilees 2004). The proteoglycan has been shown to accumulate in atherosclerotic plaques and a role in thrombosis following plaque rupture has been suggested (Kenagy et al. 2006). The cleavage of versican has been hypothesised to lead to potential regulation of cell survival and migration (Wight and Merrilees 2004). As the ADAMTS proteases have been shown to degrade versican, many hypotheses are based on this potential cleavage (Kenagy et al. 2006; Salter et al. 2010; Wight and Merrilees 2004). Very few studies have actually tested these hypotheses to try and characterise the exact cellular regulatory actions that ADAMTS proteases play during atherosclerosis (Kenagy et al. 2009; Salter et al. 2010).

Also the range of proteoglycans within the plaque is not limited to versican, other proteoglycans are present such as decorin and biglycan (Katsuda and Kaji 2003). ADAMTS proteases have been shown to cleave these proteoglycans; therefore their cleavage also may play a role in the development of atherosclerosis (Porter et al. 2005; Salter et al. 2010).

Due to the abundance of ideas relating to how ADAMTS activity could lead to the regulation of atherosclerotic progression, it was of interest to design an experimental tool that could be used to test these hypotheses. It was decided that RNAi could be utilised within many cellular assays to try and ascertain the regulatory roles of the ADAMTS proteases.

6.4.1 – RNAi

RNAi is a useful tool during the investigation of gene function in cells (Meister and Tuschl 2004). An understanding of the biological role of any gene comes only after observing the phenotypic consequences of altering the function of that gene in a living cell or organism (Paddison et al. 2002). There are two methods to introduce siRNAs into cells, you can transiently transfect dsRNA duplexes into the cell, or you can introduce a plasmid vector that encodes the shRNA to be transcribed and processed by the cellular machinery (Yu et al. 2002). Using transfection of dsRNA molecules means you can easily and quickly design target sequences (Sharp 2001). However, the transient transfection of the dsRNA means that after 48hrs <1% of the original dsRNA remains within the cell (Rao et al. 2009a; Rao et al. 2009b). Also, the higher concentration that has to be transfected increases the possibility that the dsRNA could have off-target effects within the cells (Rao et al. 2009a). The use of a plasmid delivered shRNA system can eliminate these problems as the cellular machinery continuously produces the shRNA (Rao et al. 2009b; Yu et al. 2002). Due to these disadvantages of transient transfection of dsRNA and that adenoviruses can be used to deliver shRNA into primary human cells, it was decided that the use of an adenoviral vector would be more suitable to deliver the shRNA targeting ADAMTS-1, -4 or -5.

The adenoviral vectors produced were able to silence ADAMTS-1, -4 and -5 expressions from differentiated THP-1 cells and also primary HMDM cells (Figures 6.11 and 6.12). The vectors that were produced have the potential to infect and cause gene silencing of ADAMTS-1, -4 or -5 expressions from any human cell type (Stanton et al. 2008).

The production of a vector that delivered shRNA to target ADAMTS-1, -4 or -5 provided a very useful, important tool when studying the cellular roles of ADAMTS proteases during atherosclerosis. Following the manufacture and the testing of the adenoviral vectors that were produced during this chapter, some cellular assays were designed that aimed to investigate if ADAMTS-1, -4 or -5 were involved within the regulation of that specific cellular action.

6.4.2 – Macrophage migration during atherosclerosis

Macrophage tissue infiltration is a hallmark of several pathological conditions, including atherosclerosis (Vérollet et al. 2011). However, the mechanisms involved in macrophage migration through interstitial tissues are poorly understood (Cougoule et al. 2010). Some studies suggest that macrophages migrate through a protease independent mechanism, whereas, others suggest their migration is protease dependent (Vérollet et al. 2011). This poor understanding of how macrophages migrate during pathological conditions led to investigations to study whether ADAMTS-1, -4 and -5 regulated migrations of macrophages during an *in vitro* modified Boyden chamber assay.

The modified Boyden chamber system has been used during many studies into the motility of various cell types under various conditions (Kleinman and Jacob 2001). The principle is based on the motility of a cell in response to a chemotactic concentration gradient (Kleinman and Jacob 2001). In the current assay differentiated THP-1 cells were plated onto the upper chamber and RNAi was utilised to silence ADAMTS-1, -4 and -5 expressions. The chemoattractant MCP-1 was added to the lower chamber and the cells were left to migrate for 24hrs. It was found that reduction in the expressions of ADAMTS-1, -4 and -5 had no significant effect on the migration of differentiated THP-1 cells (Figure 6.11).

There are many limitations to the current study of macrophage migration that need to be addressed in future research. Firstly, the modified Boyden chamber assay is a 2-dimensional migration assay that is better suited to study the regulation of migration through basement membranes not through 3-dimensional structures such as the ECM of

the atherosclerotic plaque (Kleinman and Jacob 2001; Vérollet et al. 2011). Also, the membrane that the cells migrated through during the assay was not coated with any ECM components. The relative lack of ECM components present during the assay would have provided no potential substrates for the ADAMTS proteases to act upon. Any regulatory actions that ADAMTS-1, -4 or -5 could have exhibited would have been very difficult to quantify using this system. One potential improvement would've been to include matrigel coating of the migratory membrane. Matrigel is a gelatinous protein mixture that represents the complex extracellular environment and is commonly used to coat the membrane in modified Boyden chamber assays (Kleinman and Jacob 2001). Some 3-dimensional migration studies have also been carried out using matrigel invasion assays, which could prove useful in these investigations (Vérollet et al. 2011). To carry out these assays, a matrigel matrix is cast around the cells of interest and their migration can be tracked using real-time filming (Vérollet et al. 2011). In previous 3-dimensional migration studies, macrophages have been shown to produce proteases, such as MMPs, that degrade the tight ECM contacts to allow migration and tunneling through the matrix (Vérollet et al. 2011). Extending these studies utilising gene silencing of ADAMTS-1, -4 and -5 by RNAi could provide some useful insights.

Another method that could be used within future studies would be the tracking of macrophage migration *in vivo*. The method used to achieve this is to study the numbers of macrophages that have migrated into the peritoneal cavity by extracting them via lavage and counting them (Park et al. 2009). One previous study used this technique and found that modified LDL reduced the migration of mouse peritoneal macrophages in a CD36-dependent manner (Park et al. 2009). They suggested that modified LDL could cause macrophage trapping within the developing atherosclerotic plaque leading to increased foam cell formation and disease progression (Park et al. 2009). It would be interesting to repeat their *in vivo* experiment with mice lacking ADAMTS-1, -4 and -5 gene activities.

The continued study of macrophage migration within atherosclerotic plaques is important, as macrophages are major players in the disease progression (McLaren et al. 2011). They are heavily involved in the initiation and maturation of the atherosclerotic

plaque and have been implicated in the expression of proteases that can destabilize the mature plaque (McLaren et al. 2011). If the understandings of the regulatory mechanisms of macrophage migration increase, then the possibility of designing therapeutics to reduce macrophage invasion and increase egress, could potentially reduce plaque size and give beneficial disease prognosis.

6.4.3 – Macrophage cholesterol uptake during atherosclerosis

Macrophage cholesterol uptake is a major event during the progression of the atherosclerotic plaque and the main mechanism of cholesterol uptake is by receptor-mediated endocytosis (McLaren et al. 2011). The main classes of receptors that are involved are called the scavenger receptors, SR-A, SR-B1 and CD-36 and their action results in the uncontrolled uptake of modified LDL leading to a lipid-laden phenotype called a macrophage foam cell (McLaren et al. 2011). Foam cell formation is a very important step during the progression of atherosclerosis; therefore the mechanisms that regulate the formation of foam cells are relatively well understood (Bobryshev 2006; McLaren et al. 2011). One area that has seen little research is that of whether ECM remodeling around the macrophage can regulate the formation of foam cells.

The experiments carried out within this chapter investigated if the reduction in expression of ADAMTS-1, -4 and -5 had a regulatory role over the uptake of modified LDL by macrophages. The experiments were carried out on differentiated THP-1 cells and primary HMDM cells and showed no change after gene silencing of ADAMTS-1, -4 or -5 (Figures 6.14 and 6.15).

One study has shown that ADAMTS-5 expression is markedly reduced in the atherosclerotic plaques of apoE null mice (Didangelos et al. 2012). The reduction in ADAMTS-5 expression was accompanied by accumulation of biglycan and versican in the ECM (Didangelos et al. 2012). When *ex vivo* experiments were carried out it was shown that ADAMTS-5 activity reduced the ability of biglycan to bind LDL and ADAMTS-5 activity was also shown to release LDL from mouse aorta sections (Didangelos et al. 2012). This is the first study that links the activity of ADAMTS proteases to the regulation of LDL

retention in the ECM. One other study has shown that the oxidation state of the LDL is also important when binding to the ECM during atherosclerosis (Wang et al. 2001). An increased level of oxidation led to more binding to the ECM (Wang et al. 2001). The study also reported that the retention of oxLDL was more pronounced in the early stages of the disease and was nearly undetectable when the plaque had matured (Wang et al. 2001).

The data from the previous studies suggest that the retention of LDL by the ECM could have a role in the formation of macrophage foam cells that is more pronounced in the very early stages of the disease (Wang et al. 2001). The retention of LDL is known to be a major initiating factor of the disease and this coupled with the trapping of macrophages caused by modified LDL in a CD36-dependent manner could go some way to explain the fast development of fatty streak lesions (Bobryshev 2006). ADAMTS-5 activity has been shown to reduce the ability of the ECM to bind LDL and therefore could be an anti-atherogenic role for the protease (Didangelos et al. 2012). However, the importance of the retention of LDL seems to reduce as the plaque matures (Wang et al. 2001). Later on in plaque progression ADAMTS proteases could have different pro-atherogenic responses, such as promotion of VSMC migration and ECM re-modelling (Salter et al. 2010; Wang et al. 2009). The understanding of the exact levels of LDL retention over the different stages of the plaque is still poorly understood and requires more research.

The LDL uptake investigations that were carried out within this chapter again have limitations similar to that observed in the last section. The *in vitro* experiments did not include any matrical components that the ADAMTS proteases could act upon. If the experiments were to be repeated then the inclusion of a matrigel component would improve the assay. Also *in vivo* experiments using apoE null mice could be utilised to investigate if the silencing of ADAMTS-1, -4 and -5 could regulate lipid burden of arteries during plaque progression.

The mechanisms of LDL retention and how ECM remodeling alters this retention, is a potentially important field of research. The understanding of the exact mechanisms by which foam cells are created in atherosclerosis will lead to more possibilities of

therapeutics that could potentially reduce foam cell formation and therefore increase disease prognosis (McLaren et al. 2011).

6.4.4 – Possible future cellular assays to utilise the ADAMTS-1, -4 and -5 shRNAs

The current study that was undertaken within this experimental chapter has yielded some very important cellular tools for the gene silencing of ADAMTS-1, -4 and -5. The work has produced shRNA vectors available to be used in a variety of different experiments that could aid the understanding of the roles that ADAMTS proteases could play in atherosclerosis.

The migration of VSMC cells during atherosclerosis is an important step in the maturation of the plaque. MMP proteases have been shown to regulate this process (Newby 2008). VSMC migration has also previously been shown to be regulated by ADAMTS-7 in neointima formation (Wang et al. 2009). The repeat of the techniques used in the previous studies such as modified Boyden chamber assays could be used in order to investigate the roles of ADAMTS-1, -4 and -5.

Another important factor involved with atherosclerosis progression is the survival of VSMC and macrophage foam cells (Newby 2006). Apoptosis and necrosis can increase the inflammatory signals that originate from the plaque and exacerbate the disease state (Lusis 2000). VSMC proliferation assays and VSMC/macrophage apoptosis assays could be carried out utilising RNAi for ADAMTS-1, -4 and -5 to ascertain how ECM remodeling can regulate cell survival. It is important to try and mimic the physiological cell-ECM contacts as much as possible. The reproduction of the exact extracellular environment is very difficult to achieve *in vitro*. Therefore the use of *in vivo* animal models is a useful tool in the research of this area (General Discussion).

Together all of these investigations should give a bigger picture of how ADAMTS proteases act within atherosclerosis and how they can be targeted in future therapeutic interventions.

Chapter 7

General Discussion

7.1 – Atherosclerosis and the ADAMTS proteases

ADAMTS proteases are a family of 19 proteins that share a similar domain pattern and substrate range; they are structurally related to the ADAM and MMP protease families and have been implicated in a number of pathophysiological conditions (Porter et al. 2005; Salter et al. 2010; Tortorella et al. 2009). The first ADAMTS family member was cloned, identified and named in a study carried out in 1997 (Kuno et al. 1997), from here the family has grown to 19 members (Porter et al. 2005; Salter et al. 2010). ADAMTS proteases are non-membrane bound proteins that act on a wide variety of ECM substrates including pro-collagen, proteoglycans and hyalectans (Jones and Riley 2005). The roles that the proteases play have been characterised during OA, evidence has been provided that suggests the proteases cleave proteoglycans such as aggrecan and COMP that leads to ECM breakdown in response to inflammatory signals from cytokines (Huang and Wu 2008; Tortorella et al. 2009). In the current study, it was of interest to compare the regulation and actions of ADAMTS proteases in relation to atherosclerosis to see if there are similarities or differences.

Atherosclerosis is considered to be a progressive disease that is characterised by lipid accumulation and inflammation within the walls of the large and medium arteries (McLaren et al. 2011). During the progression of the disease a lesion develops in the vascular wall that is called the atherosclerotic plaque. The formation of the plaque is considered to be linked with chronic inflammation, and changes to the physiological functions of the various cell types within the vascular wall (Lusis 2000). It has been recognised during clinical observations that acute symptoms usually do not occur due to the plaque critically narrowing the artery. The symptoms occur when the plaque ruptures and a thrombotic reaction is initiated (Lusis 2000). This observation has led to increasing

levels of research into what processes regulate the stability of the mature atherosclerotic plaque (Halvorsen et al. 2008). If the mechanisms that are responsible for plaque rupture are understood in more detail, it could be possible to design therapeutics to promote plaque stability and therefore reduce the prevalence of the clinical complications of CVD.

MMPs have been suggested to be major regulators of the atherosclerotic process through re-modeling of the plaque ECM leading to changes in stability (Newby et al. 2009). As the protein families are structurally related, the role for ADAMTS proteins in atherosclerosis could be similar to that of the MMPs (Salter et al. 2010). ADAMTS-1, -4, -5 and -8 are expressed within human atherosclerotic plaques, and macrophages were identified as the major contributors towards ADAMTS expression in the disease (Jonsson-Rylander et al. 2005; Lee et al. 2011; Wagsater et al. 2008). ADAMTS proteases were also expressed in VSMC and endothelial cells, but to a lower extent than macrophages and foam cells (Jonsson-Rylander et al. 2005; Wagsater et al. 2008). ADAMTS-1 expression has been studied in various wild type mouse tissues and was shown to be at the highest level in the aorta (Jonsson-Rylander et al. 2005). ADAMTS-4 mRNA was present in $LDLR^{-/-}$ $ApoB^{100/100}$ aortas before any atherosclerotic lesions were visible and the level of expression increased as the lesions became more advanced (Wagsater et al. 2008).

The above evidence suggested that ADAMTS proteases were expressed within the atherosclerotic plaque. The action of the proteases within the plaque could lead to regulation of plaque stability through various mechanisms. The current study aimed to further the understanding of the regulation of expression of ADAMTS proteases along with their roles in cellular processes associated with this disease.

7.2 – Results and Perspective

7.2.1 - Immunohistochemistry

In Chapter 3 immunohistochemistry was utilised to confirm the expression of the ADAMTS proteases within the atherosclerotic plaque. ADAMTS-1, -4 and -5 expressions were detected in human atherosclerotic plaques from carotid arteries (Figure 3.4). When looking at the distribution of ADAMTS expression it was observed that they were mainly

expressed by macrophages (Figure 3.4). The human immunohistochemical data presented in Chapter 3 confirmed findings observed by other research groups (Jonsson-Rylander et al. 2005; Lee et al. 2011; Wagsater et al. 2008). In previous studies, ADAMTS-1, -4 and -5 expressions were observed in human atherosclerotic plaques, mainly in CD68 positive macrophages (Lee et al. 2011). Also, ADAMTS-1 expression was elevated within plaques from patients with acute myocardial infarction, verses stable angina; these data suggested that ADAMTS-1 could have a regulatory role over plaque stability during atherosclerosis (Lee et al. 2011). In separate studies serum levels of ADAMTS-4 have also shown a significant correlation with the severity of coronary artery disease (Chen et al. 2011; Zha et al. 2010b). These data, taken together, outline the potential pro-atherogenic role that ADAMTS proteases could have over the stability of the atherosclerotic plaque. The data presented from the immunohistochemical analysis within this study confirmed that ADAMTS-1, -4 and -5 were being expressed within human atherosclerotic lesions. However, the mechanisms of regulation over their expression and the cellular consequences of ADAMTS action within the plaque were still relatively unknown (Salter et al. 2010). As a consequence experiments were designed to try and further the knowledge in this area, the results were presented within this thesis.

7.2.2 – THP-1 cellular model

Following on from the confirmation that ADAMTS-1, -4 and -5 were expressed in human atherosclerotic plaque; the next experimental aim was to assess how their expression was being regulated during disease progression. Due to the difficulties in consistently accessing human atherosclerotic plaque samples and primary macrophages, it was necessary to develop a cellular model so that the regulation of ADAMTS-1, -4 and -5 expressions could be studied. The established cell line THP-1 was developed in studies presented in Chapter 3 and was utilised for many experiments, all key findings were then confirmed in HMDMs (Kohro et al. 2004). The THP-1 model has previously been used to study ADAMTS expression in macrophages in other published studies (Salter et al. 2011; Wagsater et al. 2008; Worley et al. 2003). In Chapter 3, ADAMTS-1, -4 and -5 expressions were detected in THP-1 monocytes and 24hrs of PMA stimulation significantly increased

their expression, therefore this system was chosen for all future experiments (Figures 3.11 and 3.12).

7.2.3 – Cytokine regulation of ADAMTS expression in macrophages

Once the cellular model was in place it was possible to carry out a screen using important cytokines that have been implicated in having regulatory roles during atherosclerosis (Table 3.1).

7.2.3.1 – TGF-β

In Chapter 3, it was observed that ADAMTS-4 mRNA expression was significantly reduced when differentiated THP-1 macrophages were stimulated with 30ng/ml TGF-β for 24hrs (Figure 3.14). The reduction in mRNA expression of ADAMTS-4 was also translated into a protein level change (Figure 3.15). ADAMTS-1 and -5 mRNA expressions were significantly increased after the same TGF-β treatment (Figure 3.14). TGF-β has previously been shown to have a protective role during atherosclerosis as various studies on human and mouse plaques have suggested plaque-stabilising actions (Bot et al. 2009; Frutkin et al. 2009; Jiang et al. 2004). The ADAMTS-4 data obtained in Chapter 3 was consistent with an anti-atherogenic role for TGF-β in atherosclerosis, because ADAMTS-4 is regarded as pro-atherogenic (Chen et al. 2011; Salter et al. 2011; Wagsater et al. 2008; Zha et al. 2010a). However, the ADAMTS-1 and -5 data could suggest that ADAMTS proteases have gene-specific regulation and roles in atherosclerosis. ADAMTS-1 has been implicated with a pro-atherogenic role (Jonsson-Rylander et al. 2005; Lee et al. 2011) and its expression was increased by TGF-β (Chapter 3), this highlights the pleiotropic regulatory actions that TGF-β can have during gene regulation (Singh and Ramji 2006a). In a previous study ADAMTS-5 expression was reduced in the plaques of apoE$^{-/-}$ mice, which could suggest an anti-atherogenic role for the protease (Didangelos et al. 2012). In the studies presented in Chapter 3, TGF-β was shown to increase the expression of ADAMTS-5. These data suggest that as the plaque matures the levels of T_h1 cytokines could begin to persist and the levels of ADAMTS-5 expression could reduce causing the lower levels observed in the apoE$^{-/-}$ plaques (Didangelos et al. 2012). The data presented regarding the regulation of ADAMTS-1, -4 and -5 by TGF-β highlight the importance of characterising the individual

ADAMTS proteases as to their pro- or anti-atherogenic roles and how they are regulated by specific cytokines.

7.3.2.2 – IFN-γ

ADAMTS–1 mRNA expression was significantly reduced by IFN-γ stimulation (Figure 3.1). On the other hand, ADAMTS-4 and -5 mRNA expressions were not changed after THP-1 macrophages were stimulated with IFN-γ (Figure 3.17). Representative experiments were also extended to HMDMs, where reduction in expression of ADAMTS-1, but not -4 and -5, was observed (Figure 3.19). IFN-γ has a pro-inflammatory role within atherosclerosis and acts to de-stabilise the plaque, increase MMP production and reduce collagen synthesis (McLaren and Ramji 2009; Nareika et al. 2009; Newby 2007). The results presented in this chapter are not consistent with this pro-inflammatory role of IFN-γ. Previously, ADAMTS-1 has been hypothesised to accelerate plaque progression (Jonsson-Rylander et al. 2005; Lee et al. 2011), yet the expression in differentiated THP-1 cells is reduced by IFN-γ (Chapter 3). The results presented here highlight the sometimes pleiotropic actions of IFN-γ during inflammation and atherosclerosis (McLaren and Ramji 2009).

7.3.2.3 – TL1A

It was demonstrated that when differentiated THP-1 cells were stimulated with 100ng/ml of TL1A, no significant change could be observed in ADAMTS-1, -4 and -5 expressions (Figure 3.21). Activation of DR3, which is the receptor for TL1A, has been implicated in the induction of MMP-1, -9 and -13 expressions in THP-1 cells in the presence of IFN-γ (Kang et al. 2005; Kim et al. 2001). TL1A is regarded as a pro-atherogenic cytokine during disease progression (Kang et al. 2005; McLaren et al. 2010a). These data could suggest that DR3 and its ligand, TL1A has differential regulatory actions over proteases that could influence atherosclerotic plaque stability. TL1A has also been shown to have a weaker pro-atherogenic effect when acting on its own; it only regulated the expression of MMP-1, -9 and -13 when acting in combination with IFN-γ (Kang et al. 2005). The weak regulatory action of TL1A acting alone (Figure 3.21) and its ability to act together with other cytokines implicate the protease in synergistic gene regulation (Kang et al. 2005).

7.3.2.4 – IL-17A

When differentiated THP-1 cells were stimulated with 100ng/ml of IL-17A, no significant change was observed in ADAMTS-1, -4 and -5 expressions (Figure 3.24). IL-17A and its roles during atherosclerosis are controversial (Danzaki et al. 2012; Usui et al. 2012). The majority of studies suggest a pro-atherogenic role but some *in vivo* studies have produced data to challenge this suggestion (Butcher and Galkina 2011). IL-17A is a relatively weak modulator of gene expression; it could however work in combination with other cytokines to produce regulatory effects (Butcher and Galkina 2011).

7.3.2.5 – IL-33

It was demonstrated that stimulating differentiated THP-1 cells with 10ng/ml of IL-33 produced a significant reduction in the expressions of ADAMTS-1, -4 and -5 (Figure 3.27). The reduction in ADAMTS-4 expression was confirmed at the protein level utilising SDS-PAGE and western blot analysis (Figure 3.28). Representative experiments were also extended to HMDMs, where a reduction in expression of ADAMTS-1, -4 and -5 was also observed (Figure 3.29). IL-33 has been suggested to have a protective role during atherosclerosis development (Miller 2011). The cytokine acts to promote T_h2 responses, therefore slowing inflammation and stabilising the developing plaque (Miller and Liew 2011). IL-33 has been shown to reduce atherosclerosis in $apoE^{-/-}$ mice being fed a high fat diet (Miller et al. 2008). The data that has been presented in Chapter 3 were consistent with the anti-atherogenic role of the cytokine, IL-33 acted to reduce the expression of ADAMTS proteases which have mainly been implicated in ECM remodeling and destabilisation of the atherosclerotic plaque (Chen et al. 2011; Didangelos et al. 2012; Jonsson-Rylander et al. 2005; Lee et al. 2011; Salter et al. 2010; Wagsater et al. 2008; Zha et al. 2010a).

Due to IL-33 being a recently identified cytokine that acts in an anti-atherogenic manner and the consistent nature of IL-33s regulation over ADAMTS-1, -4 and -5, it was decided that the signal transduction mechanisms underlying the response should be investigated. The investigation of how IL-33 regulated gene transcription of ADAMTS-1, -4 and -5 was carried out in Chapter 5.

7.2.4 – Regulation of ADAMTS-1, -4 and -5 by cytokines in synergy

During Chapter 4 it was shown that when IFN-γ was added in combination with either TL1A or IL-17A no synergistic response was observed in relation to ADAMTS expression (Figures 4.3 and 4.5). However, when TL1A and IL-17A were added together a synergistic response was observed that resulted in the increased expressions of ADAMTS-1, -4 and -5 in differentiated THP-1 macrophages and HMDMs (Figures 4.4 and 4.6). This novel finding suggested that the synergy between TL1A and IL-17A were mainly acting in a pro-atherogenic manner when they caused an increase in ADAMTS-1, -4 and -5 expressions. Other experiments were carried out to investigate if TL1A and IL-17A could act synergistically to regulate other genes important to macrophage cell biology during atherosclerosis. The synergy was observed to be specific to ADAMTS gene regulation during our investigations as TL1A and IL-17A showed no synergistic effect on macrophage oxLDL uptake (Figure 4.9). It would be interesting to explore other aspects of macrophage cell biology to investigate if TL1A and IL-17A could act to regulate other cellular functions.

7.2.5 – Signal transduction mechanisms during IL-33 regulation of ADAMTS-1, -4 and -5 expressions

The data obtained during Chapter 5 provided a novel insight into how IL-33 regulates ADAMTS-1 and -4 expressions in macrophages. ERK-1, ERK-2, PI3Kγ, PI3Kδ, JNK and c-Jun were involved in transducing the reduction of ADAMTS-1 and -4 expressions by IL-33, but p38 and NFκB were not involved (Table 5.4). The regulation of ADAMTS-1 and -4 by IL-33 was transduced in a ST2-dependent manner. The data that was presented in Chapter 5 showed how an anti-inflammatory T_h2 cytokine, IL-33 acted to reduce the expression of ADAMTS-1 and -4.

The data provides insight into specific signalling cascades and cytokine responses that could be targeted by therapeutics in an attempt to modulate the stability of the atherosclerotic plaque through an ADAMTS dependent pathway. The signalling mechanism that transduces the attenuation of ADAMTS-4 expression by TGF-β, another anti-inflammatory cytokine has been previously investigated (Salter et al. 2011). It would be useful if a range of cytokines that acted upon ADAMTS expression were studied and

their signalling cascades were documented. The resulting data could prove interesting when comparing pro- and anti-atherogenic cytokines.

The regulation of ADAMTS expression by cytokines proves to be very complex. The regulation is gene specific and sometimes doesn't follow the classical pro- and anti-atherogenic characterisations of the cytokines. The research area requires more work to try and produce a detailed screen of cytokine regulatory patterns over the specific ADAMTS family members. If the work is carried out then it could be possible to highlight therapeutic targets that could regulate the expression of the ADAMTS through pharmacological intervention.

The data also emphasised how poor our understanding is of specific ADAMTS actions in the plaque after they have been expressed. Chapter 6 of the current thesis aimed to utilise RNAi in order to target the expression of ADAMTS-1, -4 and -5 and assess their roles within cellular assays.

7.2.6 – RNA interference of ADAMTS-1, -4 and -5

The production of a vector that delivered shRNA to target ADAMTS-1, -4 or -5 provided a very useful, important tool when studying the cellular roles of ADAMTS proteases during atherosclerosis.

In Chapter 6 differentiated THP-1 cells were plated onto the upper chamber and RNAi was utilised to silence ADAMTS-1, -4 and -5 expressions. The chemoattractant MCP-1 was added to the lower chamber and the cells were left to migrate for 24hrs. It was found that reduction in the expressions of ADAMTS-1, -4 and -5 had no significant effect on the migration of differentiated THP-1 cells (Figure 6.13).

Another experiment that was carried out within Chapter 6 investigated if the reduction in expression of ADAMTS-1, -4 and -5 had a regulatory role on the uptake of modified LDL by macrophages. The experiments were carried out on differentiated THP-1 cells and primary HMDM cells and showed no significant change after gene silencing of ADAMTS-1, -4 or -5 (Figures 6.14 and 6.15).

The macrophage migration and LDL uptake investigations that were carried out within Chapter 6 had some limitations that may have affected the results. The *in vitro* experiments did not include any ECM components that the ADAMTS proteases could act upon. If the experiments were to be repeated then the inclusion of a matrigel component would improve the assays as it would be a more accurate representation of the ECM surroundings within the atherosclerotic plaque.

7.3 – The future of ADAMTS research in Atherosclerosis

7.3.1 - Immunohistochemistry

One experiment that was not carried out during this study was to compare the expression of ADAMTS-1, -4 and -5 between healthy blood vessels and atherosclerotic blood vessels by immunohistochemistry. These experiments could have revealed the patterns of regulation of expression of the proteases during health and disease. It would have been interesting to compare these results to those that were obtained when comparing patients with stable angina and acute myocardial infarction to try and pinpoint ADAMTS proteases that have pro- or anti-atherogenic roles (Lee et al. 2011). Also, experiments within Chapter 3 that aimed to characterise the expression of ADAMTS-1, -4 and -5 in apoE$^{-/-}$ mouse atherosclerotic plaques were unsuccessful due to technical problems. It would be useful for this work to be completed in future experiments in order to aid understanding of how ADAMTS expression is regulated within a common mouse model of atherosclerosis.

7.3.2 – Possible *in vitro* assays

Cytokines have been shown to regulate the expression of the ADAMTS proteases in chrondrocytes during OA and also in macrophages during atherosclerosis (Salter et al. 2010). The data that was presented in Chapter 3 and 4 of this thesis emphasised the important role that cytokines have in the regulation of ADAMTS expression. It would be useful to use the THP-1 cellular model that was developed during this investigation to screen some more cytokines that have been shown to be active during atherosclerotic progression (Tedgui and Mallat 2006). Some examples of possible cytokines to investigate would be TNF-α, IL-1β, IL-4, IL-6 and IL-10 (Tedgui and Mallat 2006). Any interesting

regulatory patterns that are observed could then be studied as to ascertain detailed signal transduction mechanisms of the responses. The responses and mechanisms could then be assessed as to search for patterns between pro- and anti-atherogenic responses.

Another interesting experiment would be to try and assess what regulates the action of ADAMTS proteases within the atherosclerotic plaque after they have been expressed. An activity assay could be designed that utilised the addition of active recombinant ADAMTS proteins to different ECM components such as versican or aggrecan, under different conditions. The method that could be used to ascertain if the ECM components were being cleaved is by using HPLC and looking for breakdown products produced by ADAMTS cleavage products. The use of SDS-PAGE and western blotting could also be utilised with neoepitope binding antibodies (Gendron et al. 2007; Koo et al. 2007; Mort et al. 2003; Salter et al. 2010).

Extracellular regulation of ADAMTS action remains a poorly understood area. One regulatory property that is known is that the activity of ADAMTS can be regulated by the TIMPs (Salter et al. 2010). TIMPs -1, -2, -3 and -4 are endogenous inhibitors of the MMPs, however TIMP-3 has been found to be an important specific inhibitor of ADAMTS-4 and -5 (Kashiwagi et al. 2001). In addition TIMP-2 and TIMP-3 can inhibit ADAMTS-1 aggrecanase activity (Rodríguez-Manzaneque et al. 2002). Recombinant TIMPs could be added into the activity assays and the resulting regulation over ADAMTS activity could be assessed.

During Chapter 6 of this thesis an important tool was developed for use within cellular assays to assess the roles of ADAMTS action during atherosclerosis. The adenoviral vector that delivered shRNA to target ADAMTS-1, -4 and -5 can now potentially be used in many cellular assays to try and delineate the role of the ADAMTS proteases during atherosclerosis.

ADAMTS cleavage of versican has been hypothesised to have a regulatory role over the migration of VSMC during the progression of the mature atherosclerotic plaque (Salter et al. 2010). In a previous study ADAMTS-7 activity was shown to enhance the migration of

VSMC *in vitro* (Wang et al. 2009). The assays used during that study were the *in vitro* scratch-wound assay and a modified Boyden chamber coated with basement membrane components that mimicked the attachments to the cells before migration (Wang et al. 2009). These experiments would be very useful to repeat utilising the RNAi vectors that were produced during Chapter 6 of this thesis. The experiments would provide a screen of specific ADAMTS members to show if there are any family member specific regulatory actions on VSMC migration during atherosclerosis.

Another *in vitro* experiment that could be carried out is a repeat of the assays that were attempted in Chapter 6. The macrophage migration assay and the intracellular cholesterol assay could be repeated with the addition of a matrigel component. The matrigel would provide a better representation of the extracellular environment of the cells and therefore provide a substrate for the ADAMTS proteases to act upon. The regulation of cholesterol efflux could also be investigated by using radiolabelled cholesterol. These data would provide a detailed understanding of how ADAMTS proteases potentially regulate foam cell formation.

Other important processes within the development of the atherosclerotic plaque that have been implicated in the level of plaque stability are apoptosis and proliferation of cells within the plaque such as VSMC, EC and foam cells (Halvorsen et al. 2008). In order to try and study the regulatory role that the ADAMTS proteases have over these processes it is important to try and create the exact extracellular surroundings for the cells, as they would have within the plaque. This proves very difficult in practice and makes the assay very difficult to design. ADAMTS regulatory pathways rely on the cleavage of the ECM, therefore the experiments need to be carefully designed to include this component. The difficulty in accurately simulating the exact extracellular environment that cells experience within the atherosclerotic plaque is a major hurdle when designing *in vitro* assays to assess the role of ADAMTS proteases during atherosclerotic progression. One way to get over this problem would be to design *in vivo* experiments utilising common atherosclerosis experimental models, such as $apoE^{-/-}$ or $LDLR^{-/-}$ and gene silencing of the ADAMTS protease family members.

7.3.3 – Possible *in vivo* studies

The action of the ADAMTS proteases has previously been studied using *in vivo* techniques during investigations into OA (Tortorella et al. 2009). When ADAMTS-4 was silenced in mice, the growth and development appeared normal but the susceptibility to OA was unchanged (Glasson et al. 2004). ADAMTS-1 silencing resulted in mice that were slightly smaller than their WT counterparts but they were physiologically normal, their susceptibility to OA was unchanged (Little et al. 2005). However, when ADAMTS-5 was silenced the growth and development was again normal but the susceptibility to OA was reduced (Glasson et al. 2005; Majumdar et al. 2007). These data suggested that ADAMTS-5 was the predominant protease involved in the breakdown of cartilage during OA (Glasson et al. 2005). The above studies have shown that the knockout of specific ADAMTS proteases provided a viable animal model that could be reproduced to study the consequences of ADAMTS silencing during atherosclerosis progression.

In order to study how ADAMTS proteases regulate the progression of atherosclerosis it would be essential to create double knockout mice (Salter et al. 2010). Mice would be double knockout for either apoE or LDLR and ADAMTS-1, -4 or -5. Another method to study how ADAMTS proteases act within the plaque would be to transplant preselected *ex vivo* ADAMTS null bone marrow into $LDLR^{-/-}$ irradiated mice (Kalberer et al. 2000). This would allow investigation of cells from the hematopoietic lineage having ADAMTS silencing (Kalberer et al. 2000).

Once the double knockout mouse models have been produced there are multiple options for assays to investigate different regulatory actions of the ADAMTS proteases (Johnson et al. 2011). Firstly, the plaques that are produced within the atherosclerotic models could be isolated and sectioned after the required length of time being fed on a high fat diet (Johnson et al. 2011). The plaques could then be studied using microscopy related techniques and immunohistochemistry. The lesions could be characterised as to their content of VSMC, number of macrophages and amount of apoptosis by staining for common molecular markers (Johnson et al. 2011). Additionally, inflammatory status of the mice could be investigated by measuring T-cell numbers and the circulating levels of

inflammatory cytokines (Johnson et al. 2011). Potential markers that could be stained for during immunohistochemical analyses are outlined in Table 7.1.

Table 7.1: Possible markers for cells and cellular processes to be targeted by immunohistochemistry

Cell or Process to Identify	Potential Marker
VSMC number	Smooth muscle actin
Macrophage number	CD68
Apoptosis	Caspase-3

The general size, necrotic core size and an estimate of plaque stability could also be assessed from the sections (Johnson et al. 2011). The staining of whole arteries with oil red-O could also assess the general plaque burden within the animals and the lipid content of specific plaques (Gu et al. 1998). Many studies are carried out on the aorta; however brachiocephalic arteries can be sectioned and examined in order to assess the general stability of plaques: one previous study assessed the size of plaques and how many buried fibrous layers they presented in order to predict the stability of the plaques (Johnson et al. 2005).

The regulation of plaque stability by specific members of the MMP family has been investigated and the individual members have been assigned pro- or anti-atherogenic roles (Newby 2012). During the studies, low molecular weight inhibitors and gene silencing in apoE null mice reduced the actions of the MMPs (Newby 2012). When specific MMP inhibition was investigated in the apoE null background and both protective and deleterious effects were documented (Newby 2012). It has proved important that each specific MMP is characterised because they can now be specifically targeted by therapeutics depending if they are protective or problematic (Newby 2012). The work has led to some clinical trials and the area could provide some interesting future perspectives for the treatment of cardiovascular disease (Newby 2012). It would be of interest to try and extend the field of research that has been carried out for MMPs to the ADAMTS proteases.

The possibility of using transgenic gene therapy for treating human disease is still a distant possibility. However, the use of small molecule inhibitors could be a possible therapeutic option for targeting the ADAMTS proteases. ADAMTS inhibitors are available, one inhibits the activity of ADAMTS-5 and is called 5-((4-Chlorobenzylthio-3-trifluoromethyl-N-methyl-1H-pyrazol-4-yl)methylene)-2-thioxothiazolidin-4-one (Millipore). Other orally administered inhibitors of ADAMTS-4 and -5 that were designed for treatment of OA have been produced (De Savi et al. 2011). It could be possible to design more specific inhibitors for other ADAMTS family members and their therapeutic action could extend to atherosclerosis (Salter et al. 2010). Another potential mechanism to try and reduce the activity of specific ADAMTS family members could be specific monoclonal antibody targeting (Powell et al. 2007). The actions of the ADAMTS family members need to be characterised into pro- and anti-atherogenic by the use of *in vivo* mouse studies. If the research is successful then small molecule inhibitors or antibodies could possibly target the family members that prove to be pro-atherogenic. The design of these therapeutic tools could lead to new clinical trials relating to the treatment of cardiovascular disease.

7.4 – Conclusions

The current study has investigated the regulation of ADAMTS protease expression in macrophages by specific cytokines and has identified some of the underlying molecular mechanisms of signal transduction.

The cellular roles of the ADAMTS proteases within atherosclerotic disease progression remain poorly understood. During the current study some adenoviral vectors were created that deliver shRNA targeting ADAMTS-1, -4 and -5. These provide useful tools when studying the cellular roles of ADAMTS proteases in atherosclerosis. However, any future *in vitro* assays need to be carefully designed to replicate the exact extracellular substrates that the ADAMTS proteases encounter under normal physiological conditions.

One way to get around these limitations is to investigate the cellular roles of the ADAMTS proteases by *in vivo* gene silencing. The study of ADAMTS proteases in relation to

atherosclerosis will require a double knockout model comprising either the apoE null model or the LDLR null model.

The field of research is now growing and it provides some exciting opportunities for novel therapeutics of the future. The benefits of understanding how the stability of the atherosclerotic plaque is regulated can only translate into better therapeutics to regulate the stability of the plaque through pharmacological intervention.

References

Aggarwal, S. and Gurney, A. L. (2002). IL-17: prototype member of an emerging cytokine family. *J Leukoc Biol* **71:**1-8.

Aguilera, C. M., George, S. J., Johnson, J. L. and Newby, A. C. (2003). Relationship between type IV collagen degradation, metalloproteinase activity and smooth muscle cell migration and proliferation in cultured human saphenous vein. *Cardiovasc Res* **58:**679-688.

Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W. and Martin, M. U. (2007). IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc Natl Acad Sci U S A* **104:**18660-18665.

Auwerx, J. (1991). The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia* **47:**22-31.

Auwerx, J. H., Deeb, S., Brunzell, J. D., Wolfbauer, G. and Chait, A. (1989). Lipoprotein lipase gene expression in THP-1 cells. *Biochemistry* **28:**4563-4567.

Bailly, S., Brand, C., Chambaz, E. M. and Feige, J. J. (1997). Analysis of small latent transforming growth factor-beta complex formation and dissociation by surface plasmon resonance. Absence of direct interaction with thrombospondins. *J Biol Chem* **272:**16329-16334.

Bamias, G., Martin, C., Marini, M., Hoang, S., Mishina, M., Ross, W. G., Sachedina, M. A. *et al.* (2003). Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. *J Immunol* **171:**4868-4874.

Barin, J. G., Christian Baldeviano, G., Talor, M. V., Wu, L., Ong, S., Quader, F., Chen, P. *et al.* (2011). Macrophages participate in IL-17-mediated inflammation. *Eur J Immunol*.

Basheeruddin, K., Rechtoris, C. and Mazzone, T. (1992). Transcriptional and post-transcriptional control of apolipoprotein E gene expression in differentiating human monocytes. *J Biol Chem* **267:**1219-1224.

Bennett, M. R. (1999). Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture. *Cardiovasc Res* **41:**361-368.

Bewig, B. and Schmidt, W. E. (2000). Accelerated titering of adenoviruses. *Biotechniques* **28:**870-873.

Bliss, S. P., Miller, A., Navratil, A. M., Xie, J., McDonough, S. P., Fisher, P. J., Landreth, G. E. *et al.* (2009). ERK signaling in the pituitary is required for female but not male fertility. *Mol Endocrinol* **23:**1092-1101.

Bobryshev, Y. V. (2006). Monocyte recruitment and foam cell formation in atherosclerosis. *Micron* **37:**208-222.

Bond, M., Chase, A. J., Baker, A. H. and Newby, A. C. (2001). Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. *Cardiovasc Res* **50:**556-565.

Bot, P. T., Hoefer, I. E., Sluijter, J. P., van Vliet, P., Smits, A. M., Lebrin, F., Moll, F. *et al.* (2009). Increased expression of the transforming growth factor-beta signaling pathway, endoglin, and early growth response-1 in stable plaques. *Stroke* **40:**439-447.

Boyle, J. J., Johns, M., Kampfer, T., Nguyen, A. T., Game, L., Schaer, D. J., Mason, J. C. *et al.* (2012). Activating transcription factor 1 directs Mhem atheroprotective macrophages through coordinated iron handling and foam cell protection. *Circ Res* **110:**20-33.

Brand, K., Mackman, N. and Curtiss, L. K. (1993). Interferon-gamma inhibits macrophage apolipoprotein E production by posttranslational mechanisms. *J Clin Invest* **91:**2031-2039.

Brint, E. K., Fitzgerald, K. A., Smith, P., Coyle, A. J., Gutierrez-Ramos, J. C., Fallon, P. G. and O'Neill, L. A. (2002). Characterization of signaling pathways activated by the interleukin 1 (IL-1) receptor homologue T1/ST2. A role for Jun N-terminal kinase in IL-4 induction. *J Biol Chem* **277:**49205-49211.

Butcher, M. and Galkina, E. (2011). Current views on the functions of interleukin-17A-producing cells in atherosclerosis. *Thromb Haemost* **106:**787-795.

Butcher, M. J., Gjurich, B. N., Phillips, T. and Galkina, E. V. (2012). The IL-17A/IL-17RA Axis Plays a Proatherogenic Role via the Regulation of Aortic Myeloid Cell Recruitment. *Circ Res* **110:**675-687.

Cal, S., Obaya, A. J., Llamazares, M., Garabaya, C., Quesada, V. and López-Otín, C. (2002). Cloning, expression analysis, and structural characterization of seven novel human ADAMTSs, a family of metalloproteinases with disintegrin and thrombospondin-1 domains. *Gene* **283:**49-62.

Charo, I. F. and Taubman, M. B. (2004). Chemokines in the pathogenesis of vascular disease. *Circ Res* **95:**858-866.

Chawla, A. (2010). Control of macrophage activation and function by PPARs. *Circ Res* **106:**1559-1569.

Chen, L., Yang, L., Zha, Y. and Cui, L. (2011). Association of serum a disintegrin and metalloproteinase with thrombospodin motif 4 levels with the presence and severity of coronary artery disease. *Coron Artery Dis* **22:**570-576.

Cheng, K., Xie, G. and Raufman, J. P. (2007). Matrix metalloproteinase-7-catalyzed release of HB-EGF mediates deoxycholyltaurine-induced proliferation of a human colon cancer cell line. *Biochem Pharmacol* **73:**1001-1012.

Cheng, X., Taleb, S., Wang, J., Tang, T. T., Chen, J., Gao, X. L., Yao, R. *et al.* (2011). Inhibition of IL-17A in atherosclerosis. *Atherosclerosis* **215:**471-474.

Chinetti-Gbaguidi, G. and Staels, B. (2009). Lipid ligand-activated transcription factors regulating lipid storage and release in human macrophages. *Biochim Biophys Acta* **1791:**486-493.

Choi, W. S., Jeon, O. H., Kim, H. H. and Kim, D. S. (2008). MMP-2 regulates human platelet activation by interacting with integrin alphaIIbbeta3. *J Thromb Haemost* **6:**517-523.

Choi, Y. S., Choi, H. J., Min, J. K., Pyun, B. J., Maeng, Y. S., Park, H., Kim, J. *et al.* (2009). Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood* **114:**3117-3126.

Chung, H. K., Lee, I. K., Kang, H., Suh, J. M., Kim, H., Park, K. C., Kim, D. W. *et al.* (2002). Statin inhibits interferon-gamma-induced expression of intercellular adhesion molecule-1 (ICAM-1) in vascular endothelial and smooth muscle cells. *Exp Mol Med* **34:**451-461.

Chung, K. F. (2011). p38 mitogen-activated protein kinase pathways in asthma and COPD. *Chest* **139:**1470-1479.

Chung, K. H., Hart, C. C., Al-Bassam, S., Avery, A., Taylor, J., Patel, P. D., Vojtek, A. B. *et al.* (2006). Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res* **34:**e53.

Clarke, M. and Bennett, M. (2006). The emerging role of vascular smooth muscle cell apoptosis in atherosclerosis and plaque stability. *American Journal of Nephrology* **26:**531-535.

Clarke, M. C., Littlewood, T. D., Figg, N., Maguire, J. J., Davenport, A. P., Goddard, M. and Bennett, M. R. (2008). Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res* **102:**1529-1538.

Cohen, P. (2010). Guidelines for the effective use of chemical inhibitors of protein function to understand their roles in cell regulation. *Biochem J* **425:**53-54.

Corps, A. N., Jones, G. C., Harrall, R. L., Curry, V. A., Hazleman, B. L. and Riley, G. P. (2008). The regulation of aggrecanase ADAMTS-4 expression in human Achilles tendon and tendon-derived cells. *Matrix Biol* **27:**393-401.

Corrado, E., Rizzo, M., Coppola, G., Fattouch, K., Novo, G., Marturana, I., Ferrara, F. *et al.* (2010). An update on the role of markers of inflammation in atherosclerosis. *J Atheroscler Thromb* **17:**1-11.

Coughlan, T. C., Crawford, A., Goldring, M. B., Hatton, P. V. and Barker, M. D. (2010). Lentiviral shRNA knock-down of ADAMTS-5 and -9 restores matrix deposition in 3D chondrocyte culture. *J Tissue Eng Regen Med* **4:**611-618.

Cougoule, C., Le Cabec, V., Poincloux, R., Al Saati, T., Mège, J. L., Tabouret, G., Lowell, C. A. *et al.* (2010). Three-dimensional migration of macrophages requires Hck for podosome organization and extracellular matrix proteolysis. *Blood* **115:**1444-1452.

Cross, N. A., Chandrasekharan, S., Jokonya, N., Fowles, A., Hamdy, F. C., Buttle, D. J. and Eaton, C. L. (2005). The expression and regulation of ADAMTS-1, -4, -5, -9, and -15, and TIMP-3 by TGFbeta1 in prostate cells: relevance to the accumulation of versican. *Prostate* **63:**269-275.

Dalby, B., Cates, S., Harris, A., Ohki, E. C., Tilkins, M. L., Price, P. J. and Ciccarone, V. C. (2004). Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods* **33:**95-103.

Danzaki, K., Matsui, Y., Ikesue, M., Ohta, D., Ito, K., Kanayama, M., Kurotaki, D. *et al.* (2012). Interleukin-17A deficiency accelerates unstable atherosclerotic plaque formation in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **32:**273-280.

de Boer, O. J., van der Meer, J. J., Teeling, P., van der Loos, C. M., Idu, M. M., van Maldegem, F., Aten, J. *et al.* (2009). Differential expression of interleukin-17 family cytokines in intact and complicated human atherosclerotic plaques. *Journal of Pathology*.

De Savi, C., Pape, A., Sawyer, Y., Milne, D., Davies, C., Cumming, J. G., Ting, A. *et al.* (2011). Orally active achiral N-hydroxyformamide inhibitors of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) for the treatment of osteoarthritis. *Bioorg Med Chem Lett* **21:**3301-3306.

Demircan, K., Hirohata, S., Nishida, K., Hatipoglu, O. F., Oohashi, T., Yonezawa, T., Apte, S. S. *et al.* (2005). ADAMTS-9 is synergistically induced by interleukin-1beta and tumor necrosis factor alpha in OUMS-27 chondrosarcoma cells and in human chondrocytes. *Arthritis Rheum* **52:**1451-1460.

Dhillon, A. S., Hagan, S., Rath, O. and Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene* **26:**3279-3290.

Didangelos, A., Mayr, U., Monaco, C. and Mayr, M. (2012). Novel Role of ADAMTS-5 Protein in Proteoglycan Turnover and Lipoprotein Retention in Atherosclerosis. *J Biol Chem* **287:**19341-19345.

Ding, H. S., Yang, J., Ding, J. W., Chen, P. and Zhu, P. (2012). Interleukin-17 contributes to cardiovascular diseases. *Mol Biol Rep* **39:**7473-7478.

Doran, A. C., Meller, N. and McNamara, C. A. (2008). Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* **28:**812-819.

Draude, G. and Lorenz, R. L. (2000). TGF-beta1 downregulates CD36 and scavenger receptor A but upregulates LOX-1 in human macrophages. *Am J Physiol Heart Circ Physiol* **278:**H1042-1048.

Dwivedi, A., Slater, S. C. and George, S. J. (2009). MMP-9 and -12 cause N-cadherin shedding and thereby beta-catenin signalling and vascular smooth muscle cell proliferation. *Cardiovasc Res* **81:**178-186.

Eggert, U. S., Field, C. M. and Mitchison, T. J. (2006). Small molecules in an RNAi world. *Mol Biosyst* **2:**93-96.

Eid, R. E., Rao, D. A., Zhou, J., Lo, S. F., Ranjbaran, H., Gallo, A., Sokol, S. I. *et al.* (2009). Interleukin-17 and interferon-gamma are produced concomitantly by human coronary artery-infiltrating T cells and act synergistically on vascular smooth muscle cells. *Circulation* **119:**1424-1432.

Elgharib, N., Chi, D. S., Younis, W., Wehbe, S. and Krishnaswamy, G. (2003). C-reactive protein as a novel biomarker. Reactant can flag atherosclerosis and help predict cardiac events. *Postgrad Med* **114:**39-44; quiz 16.

Evanko, S. P., Angello, J. C. and Wight, T. N. (1999). Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **19:**1004-1013.

Foldager, C. B., Munir, S., Ulrik-Vinther, M., Søballe, K., Bünger, C. and Lind, M. (2009). Validation of suitable house keeping genes for hypoxia-cultured human chondrocytes. *BMC Mol Biol* **10:**94.

Fowlkes, J. L., Enghild, J. J., Suzuki, K. and Nagase, H. (1994). Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. *J Biol Chem* **269:**25742-25746.

Fowlkes, J. L., Thrailkill, K. M., Serra, D. M., Suzuki, K. and Nagase, H. (1995). Matrix metalloproteinases as insulin-like growth factor binding protein-degrading proteinases. *Prog Growth Factor Res* **6:**255-263.

Frutkin, A. D., Otsuka, G., Stempien-Otero, A., Sesti, C., Du, L., Jaffe, M., Dichek, H. L. *et al.* (2009). TGF-[beta]1 limits plaque growth, stabilizes plaque structure, and prevents aortic dilation in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* **29:**1251-1257.

Funakoshi-Tago, M., Tago, K., Hayakawa, M., Tominaga, S., Ohshio, T., Sonoda, Y. and Kasahara, T. (2008). TRAF6 is a critical signal transducer in IL-33 signaling pathway. *Cell Signal* **20:**1679-1686.

Funakoshi-Tago, M., Tago, K., Sato, Y., Tominaga, S. and Kasahara, T. (2011). JAK2 is an important signal transducer in IL-33-induced NF-κB activation. *Cell Signal* **23:**363-370.

Gendron, C., Kashiwagi, M., Lim, N. H., Enghild, J. J., Thøgersen, I. B., Hughes, C., Caterson, B. *et al.* (2007). Proteolytic activities of human ADAMTS-5: comparative studies with ADAMTS-4. *J Biol Chem* **282:**18294-18306.

Geng, Y. J. and Hansson, G. K. (1992). Interferon-gamma inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. *J Clin Invest* **89:**1322-1330.

Ginzinger, D. G. (2002). Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology* **30:**503-512.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. and Mathieu, C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25:**386-401.

Glasson, S. S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H. L., Flannery, C. R. *et al.* (2005). Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature* **434:**644-648.

Glasson, S. S., Askew, R., Sheppard, B., Carito, B. A., Blanchet, T., Ma, H. L., Flannery, C. R. *et al.* (2004). Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. *Arthritis Rheum* **50:**2547-2558.

Gleissner, C. A., Shaked, I., Little, K. M. and Ley, K. (2010). CXC chemokine ligand 4 induces a unique transcriptome in monocyte-derived macrophages. *J Immunol* **184:**4810-4818.

Goliasch, G., Wiesbauer, F., Kastl, S. P., Katsaros, K. M., Blessberger, H., Maurer, G., Schillinger, M. *et al.* (2012). Premature myocardial infarction is associated with low serum levels of Wnt-1. *Atherosclerosis*.

Gordon, S. and Martinez, F. O. (2010). Alternative activation of macrophages: mechanism and functions. *Immunity* **32:**593-604.

Greenow, K., Pearce, N. J. and Ramji, D. P. (2005). The key role of apolipoprotein E in atherosclerosis. *J Mol Med (Berl)* **83:**329-342.

Gu, L., Okada, Y., Clinton, S. K., Gerard, C., Sukhova, G. K., Libby, P. and Rollins, B. J. (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* **2:**275-281.

Gu, Y., Hu, X., Liu, C., Qv, X. and Xu, C. (2008). Interleukin (IL)-17 promotes macrophages to produce IL-8, IL-6 and tumour necrosis factor-alpha in aplastic anaemia. *British journal of haematology* **142:**109-114.

Gupta, S., Pablo, A. M., Jiang, X., Wang, N., Tall, A. R. and Schindler, C. (1997). IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest* **99:**2752-2761.

Halvorsen, B., Otterdal, K., Dahl, T. B., Skjelland, M., Gullestad, L., Oie, E. and Aukrust, P. (2008). Atherosclerotic plaque stability - What determines the fate of the plaque? *Progress in Cardiovascular Diseases* **51:**183-194.

Harrington, L. E., Mangan, P. R. and Weaver, C. T. (2006). Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr Opin Immunol* **18:**349-356.

Hartley, J. W., Evans, L. H., Green, K. Y., Naghashfar, Z., Macias, A. R., Zerfas, P. M. and Ward, J. M. (2008). Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology* **5:**1.

Heinonen, H., Nieminen, A., Saarela, M., Kallioniemi, A., Klefstrom, J., Hautaniemi, S. and Monni, O. (2008). Deciphering downstream gene targets of PI3K/mTOR/p70S6K pathway in breast cancer.*BMC Genomics*. Vol. 9. England, p. 348.

Hirata, Y., Tabata, M., Kurobe, H., Motoki, T., Akaike, M., Nishio, C., Higashida, M. *et al.* (2011). Coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue. *J Am Coll Cardiol* **58:**248-255.

Hirose, J., Kawashima, H., Yoshie, O., Tashiro, K. and Miyasaka, M. (2001). Versican interacts with chemokines and modulates cellular responses. *J Biol Chem* **276:**5228-5234.

Hot, A., Lenief, V. and Miossec, P. (2012). Combination of IL-17 and TNFα induces a pro-inflammatory, pro-coagulant and pro-thrombotic phenotype in human endothelial cells. *Ann Rheum Dis* **71:**768-776.

Hsu, D. W., Hakim, F., Biller, B. M., de la Monte, S., Zervas, N. T., Klibanski, A. and Hedley-Whyte, E. T. (1993). Significance of proliferating cell nuclear antigen index in predicting pituitary adenoma recurrence. *J Neurosurg* **78:**753-761.

Hsu, H. and Viney, J. L. (2011). The tale of TL1A in inflammation. *Mucosal Immunol* **4:**368-370.

Huang, K. and Wu, L. D. (2008). Aggrecanase and aggrecan degradation in osteoarthritis: a review. *J Int Med Res* **36:**1149-1160.

Hughes, T. R., Tengku-Muhammad, T. S., Irvine, S. A. and Ramji, D. P. (2002). A novel role of Sp1 and Sp3 in the interferon-gamma -mediated suppression of macrophage lipoprotein lipase gene transcription. *J Biol Chem* **277:**11097-11106.

Hui, W., Barksby, H. E., Young, D. A., Cawston, T. E., McKie, N. and Rowan, A. D. (2005). Oncostatin M in combination with tumour necrosis factor {alpha} induces a chondrocyte membrane associated aggrecanase that is distinct from ADAMTS aggrecanase-1 or -2. *Ann Rheum Dis* **64:**1624-1632.

Ikonen, E. (2006). Mechanisms for Cellular Transport: Defects and Human Disease. *Physiological Reviews* **86:**1237-1261.

Ilić, D., Almeida, E. A., Schlaepfer, D. D., Dazin, P., Aizawa, S. and Damsky, C. H. (1998). Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* **143:**547-560.

Imaizumi, K. (2011). Diet and atherosclerosis in apolipoprotein E-deficient mice. *Biosci Biotechnol Biochem* **75:**1023-1035.

Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E. and Herz, J. (1993). Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* **92:**883-893.

Ishibashi, S., Goldstein, J. L., Brown, M. S., Herz, J. and Burns, D. K. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest* **93:**1885-1893.

Jiang, X., Zeng, H. S., Guo, Y., Zhou, Z. B., Tang, B. S. and Li, F. K. (2004). The expression of matrix metalloproteinases-9, transforming growth factor-beta1 and transforming growth factor-beta receptor I in human atherosclerotic plaque and their relationship with plaque stability. *Chin Med J (Engl)* **117:**1825-1829.

Johnson, J. L., Devel, L., Czarny, B., George, S. J., Jackson, C. L., Rogakos, V., Beau, F. *et al.* (2011). A selective matrix metalloproteinase-12 inhibitor retards atherosclerotic plaque development in apolipoprotein E-knockout mice. *Arterioscler Thromb Vasc Biol* **31:**528-535.

Johnson, J. L., George, S. J., Newby, A. C. and Jackson, C. L. (2005). Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc Natl Acad Sci U S A* **102:**15575-15580.

Johnson, J. L., Sala-Newby, G. B., Ismail, Y., Aguilera, C. M. and Newby, A. C. (2008). Low tissue inhibitor of metalloproteinases 3 and high matrix metalloproteinase 14 levels

defines a subpopulation of highly invasive foam-cell macrophages. *Arterioscler Thromb Vasc Biol* **28:**1647-1653.

Jonasson, L., Hansson, G. K., Bondjers, G., Noe, L. and Etienne, J. (1990). Interferon-gamma inhibits lipoprotein lipase in human monocyte-derived macrophages. *Biochim Biophys Acta* **1053:**43-48.

Jones, G. C. and Riley, G. P. (2005). ADAMTS proteinases: a multi-domain, multi-functional family with roles in extracellular matrix turnover and arthritis. *Arthritis Res Ther* **7:**160-169.

Jonsson-Rylander, A. C., Nilsson, T., Fritsche-Danielson, R., Hammarstrom, A., Behrendt, M., Andersson, J. O., Lindgren, K. *et al.* (2005). Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican. *Arterioscler Thromb Vasc Biol* **25:**180-185.

Jovanovic, D. V., Di Battista, J. A., Martel-Pelletier, J., Jolicoeur, F. C., He, Y., Zhang, M., Mineau, F. *et al.* (1998). IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* **160:**3513-3521.

Kajinami, K. and Kawai, Y. (2011). Beyond C-reactive protein; new evidence for another inflammatory biomarker predicting cardiovascular disease risk. *Atherosclerosis* **214:**39-40.

Kakkar, R., Hei, H., Dobner, S. and Lee, R. T. (2012). Interleukin 33 as a mechanically responsive cytokine secreted by living cells. *J Biol Chem* **287:**6941-6948.

Kakkar, R. and Lee, R. T. (2008). The IL33/ST2 pathway: therapeutic target and novel biomarker. *Nature Reviews Drug Discovery* **7:**827-840.

Kalberer, C. P., Pawliuk, R., Imren, S., Bachelot, T., Takekoshi, K. J., Fabry, M., Eaves, C. J. *et al.* (2000). Preselection of retrovirally transduced bone marrow avoids subsequent stem cell gene silencing and age-dependent extinction of expression of human beta-globin in engrafted mice. *Proc Natl Acad Sci U S A* **97:**5411-5415.

Kamada, N., Hisamatsu, T., Honda, H., Kobayashi, T., Chinen, H., Takayama, T., Kitazume, M. T. *et al.* (2010). TL1A produced by lamina propria macrophages induces Th1 and Th17 immune responses in cooperation with IL-23 in patients with Crohn's disease. *Inflamm Bowel Dis* **16:**568-575.

Kamekura, R., Kojima, T., Takano, K., Go, M., Sawada, N. and Himi, T. (2012). The role of IL-33 and its receptor ST2 in human nasal epithelium with allergic rhinitis. *Clin Exp Allergy* **42:**218-228.

Kang, Y., Kim, W., Bae, H., Kim, D., Park, Y. B., Park, J., Kwon, B. S. *et al.* (2005). Involvement of TL1A and DR3 in induction of
pro-inflammatory cytokines and matrix

metalloproteinase-9 in atherogenesis. *Cytokine* **29:**229-235.

Kashiwagi, M., Tortorella, M., Nagase, H. and Brew, K. (2001). TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). *J Biol Chem* **276:**12501-12504.

Katsuda, S. and Kaji, T. (2003). Atherosclerosis and extracellular matrix. *J Atheroscler Thromb* **10:**267-274.

Kawashima, H., Hirose, M., Hirose, J., Nagakubo, D., Plaas, A. H. and Miyasaka, M. (2000). Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. *J Biol Chem* **275:**35448-35456.

Kenagy, R. D., Min, S. K., Clowes, A. W. and Sandy, J. D. (2009). Cell death-associated ADAMTS4 and versican degradation in vascular tissue. *J Histochem Cytochem* **57:**889-897.

Kenagy, R. D., Plaas, A. H. and Wight, T. N. (2006). Versican degradation and vascular disease. *Trends Cardiovasc Med* **16:**209-215.

Khallou-Laschet, J., Varthaman, A., Fornasa, G., Compain, C., Gaston, A. T., Clement, M., Dussiot, M. *et al.* (2010). Macrophage plasticity in experimental atherosclerosis. *PLoS One* **5:**e8852.

Kim, E. K. and Choi, E. J. (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta* **1802:**396-405.

Kim, S. H., Lee, W. H., Kwon, B. S., Oh, G. T., Choi, Y. H. and Park, J. E. (2001). Tumor necrosis factor receptor superfamily 12 may destabilize atherosclerotic plaques by inducing matrix metalloproteinases. *Jpn Circ J* **65:**136-138.

Kleinman, H. K. and Jacob, K. (2001). Invasion assays. *Curr Protoc Cell Biol* **Chapter 12:**Unit 12.12.

Koga, M., Kai, H., Yasukawa, H., Yamamoto, T., Kawai, Y., Kato, S., Kusaba, K. *et al.* (2007). Inhibition of progression and stabilization of plaques by postnatal interferon-gamma function blocking in ApoE-knockout mice. *Circ Res* **101:**348-356.

Kohro, T., Tanaka, T., Murakami, T., Wada, Y., Aburatani, H., Hamakubo, T. and Kodama, T. (2004). A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophages. *Journal of Atherosclerosis and Thrombosis* **11:**88-97.

Kolls, J. K. and Linden, A. (2004). Interleukin-17 family members and inflammation. *Immunity* **21:**467-476.

Koo, B. H., Longpré, J. M., Somerville, R. P., Alexander, J. P., Leduc, R. and Apte, S. S. (2007). Regulation of ADAMTS9 secretion and enzymatic activity by its propeptide. *J Biol Chem* **282:**16146-16154.

Koshman, Y. E., Chu, M., Engman, S. J., Kim, T., Iyengar, R., Robia, S. L. and Samarel, A. M. (2011). Focal adhesion kinase-related nonkinase inhibits vascular smooth muscle cell invasion by focal adhesion targeting, tyrosine 168 phosphorylation, and competition for p130(Cas) binding. *Arterioscler Thromb Vasc Biol* **31:**2432-2440.

Kuno, K., Kanada, N., Nakashima, E., Fujiki, F., Ichimura, F. and Matsushima, K. (1997). Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene. *J Biol Chem* **272:**556-562.

Kurowska-Stolarska, M., Hueber, A., Stolarski, B. and McInnes, I. B. (2011). Interleukin-33: a novel mediator with a role in distinct disease pathologies. *J Intern Med* **269:**29-35.

Lee, C. W., Hwang, I., Park, C. S., Lee, H., Park, D. W., Kang, S. J., Lee, S. H. *et al.* (2011). Comparison of ADAMTS-1, -4 and -5 expression in culprit plaques between acute myocardial infarction and stable angina. *J Clin Pathol* **64:**399-404.

Legastelois, I., Levrey, H., Greenland, T., Mornex, J. F. and Cordier, G. (1998). Visna-maedi virus induces interleukin-8 in sheep alveolar macrophages through a tyrosine-kinase signaling pathway. *Am J Respir Cell Mol Biol* **18:**532-537.

Lemire, J. M., Chan, C. K., Bressler, S., Miller, J., LeBaron, R. G. and Wight, T. N. (2007). Interleukin-1beta selectively decreases the synthesis of versican by arterial smooth muscle cells. *J Cell Biochem* **101:**753-766.

Levkau, B., Kenagy, R. D., Karsan, A., Weitkamp, B., Clowes, A. W., Ross, R. and Raines, E. W. (2002). Activation of metalloproteinases and their association with integrins: an auxiliary apoptotic pathway in human endothelial cells. *Cell Death Differ* **9:**1360-1367.

Li, H., Cybulsky, M. I., Gimbrone, M. A. and Libby, P. (1993). Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. *Am J Pathol* **143:**1551-1559.

Li, N., McLaren, J. E., Michael, D. R., Clement, M., Fielding, C. A. and Ramji, D. P. (2010). ERK is integral to the IFN-γ-mediated activation of STAT1, the expression of key genes implicated in atherosclerosis, and the uptake of modified lipoproteins by human macrophages. *J Immunol* **185:**3041-3048.

Li, Q. and Verma, I. M. (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol* **2:**725-734.

Liang, S. C., Tan, X. Y., Luxenberg, D. P., Karim, R., Dunussi-Joannopoulos, K., Collins, M. and Fouser, L. A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* **203:**2271-2279.

Libby, P. (2002). Inflammation in atherosclerosis. *Nature* **420:**868-874.

Libby, P. and Aikawa, M. (2002). Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. *Nat Med* **8:**1257-1262.

Little, C. B., Mittaz, L., Belluoccio, D., Rogerson, F. M., Campbell, I. K., Meeker, C. T., Bateman, J. F. *et al.* (2005). ADAMTS-1-knockout mice do not exhibit abnormalities in aggrecan turnover in vitro or in vivo. *Arthritis Rheum* **52:**1461-1472.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:**402-408.

Llorente-Cortés, V., Otero-Viñas, M., Hurt-Camejo, E., Martínez-González, J. and Badimon, L. (2002). Human coronary smooth muscle cells internalize versican-modified LDL through LDL receptor-related protein and LDL receptors. *Arterioscler Thromb Vasc Biol* **22:**387-393.

Luan, Y., Kong, L., Howell, D. R., Ilalov, K., Fajardo, M., Bai, X. H., Di Cesare, P. E. *et al.* (2008). Inhibition of ADAMTS-7 and ADAMTS-12 degradation of cartilage oligomeric matrix protein by alpha-2-macroglobulin. *Osteoarthritis Cartilage* **16:**1413-1420.

Lusis, A. J. (2000). Atherosclerosis. *Nature* **407:**233-241.

Lusis, A. J., Mar, R. and Pajukanta, P. (2004). Genetics of atherosclerosis. *Annual Review of Genomics and Human Genetics* **5:**189-218.

Lutgens, E. and Daemen, M. J. (2001). Transforming growth factor-beta: a local or systemic mediator of plaque stability? *Circ Res* **89:**853-855.

Luzina, I. G., Pickering, E. M., Kopach, P., Kang, P. H., Lockatell, V., Todd, N. W., Papadimitriou, J. C. *et al.* (2012). Full-Length IL-33 Promotes Inflammation but not Th2 Response In Vivo in an ST2-Independent Fashion. *J Immunol* **189:**403-410.

Mach, F., Schönbeck, U., Bonnefoy, J. Y., Pober, J. S. and Libby, P. (1997a). Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. *Circulation* **96:**396-399.

Mach, F., Schönbeck, U., Sukhova, G. K., Bourcier, T., Bonnefoy, J. Y., Pober, J. S. and Libby, P. (1997b). Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci U S A* **94:**1931-1936.

Maess, M. B., Sendelbach, S. and Lorkowski, S. (2010). Selection of reliable reference genes during THP-1 monocyte differentiation into macrophages. *BMC Mol Biol* **11:**90.

Majumdar, M. K., Askew, R., Schelling, S., Stedman, N., Blanchet, T., Hopkins, B., Morris, E. A. *et al.* (2007). Double-knockout of ADAMTS-4 and ADAMTS-5 in mice results in physiologically normal animals and prevents the progression of osteoarthritis. *Arthritis Rheum* **56:**3670-3674.

Mallat, Z., Taleb, S., Ait-Oufella, H. and Tedgui, A. (2009). The role of adaptive T cell immunity in atherosclerosis. *J Lipid Res* **50 Suppl:**S364-369.

Mandel, M. and Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. *J Mol Biol* **53:**159-162.

Manning, B. D. and Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell* **129:**1261-1274.

Martinez, F. O. (2011). Regulators of macrophage activation. *Eur J Immunol* **41:**1531-1534.

Martinez, F. O., Helming, L. and Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* **27:**451-483.

McLaren, J. E., Calder, C. J., McSharry, B. P., Sexton, K., Salter, R. C., Singh, N. N., Wilkinson, G. W. *et al.* (2010a). The TNF-like protein 1A-death receptor 3 pathway promotes macrophage foam cell formation in vitro. *J Immunol* **184:**5827-5834.

McLaren, J. E., Michael, D. R., Ashlin, T. G. and Ramji, D. P. (2011). Cytokines, macrophage lipid metabolism and foam cells: implications for cardiovascular disease therapy. *Prog Lipid Res* **50:**331-347.

McLaren, J. E., Michael, D. R., Salter, R. C., Ashlin, T. A., Clader, C. J., Miller, A. M., Liew, F. Y. *et al.* (2010b). IL-33 reduces macrophage foam cell formation. *Journal of Immunology*.

McLaren, J. E., Michael, D. R., Salter, R. C., Ashlin, T. G., Calder, C. J., Miller, A. M., Liew, F. Y. *et al.* (2010c). IL-33 reduces macrophage foam cell formation. *J Immunol* **185:**1222-1229.

McLaren, J. E. and Ramji, D. P. (2009). Interferon gamma: a master regulator of atherosclerosis. *Cytokine Growth Factor Rev* **20:**125-135.

Meister, G. and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* **431:**343-349.

Michael, D. R., Salter, R. C. and Ramji, D. P. (2012). TGF-β inhibits the uptake of modified low density lipoprotein by human macrophages through a Smad-dependent pathway: A dominant role for Smad-2. *Biochim Biophys Acta*.

Migone, T., Zhang, J., Luo, X., Zhuang, L., Chen, C., Hu, B., Hong, J. S. *et al.* (2002). TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* **16:**479-492.

Miller, A. M. (2011). Role of IL-33 in inflammation and disease. *J Inflamm (Lond)* **8:**22.

Miller, A. M. and Liew, F. Y. (2011). The IL-33/ST2 pathway--A new therapeutic target in cardiovascular disease. *Pharmacol Ther* **131:**179-186.

Miller, A. M., Xu, D., Asquith, D. L., Denby, L., Li, Y., Sattar, N., Baker, A. H. *et al.* (2008). IL-33 reduces the development of atherosclerosis. *J Exp Med* **205:**339-346.

Miller, Y. I., Choi, S. H., Fang, L. and Tsimikas, S. (2010). Lipoprotein modification and macrophage uptake: role of pathologic cholesterol transport in atherogenesis. *Subcell Biochem* **51:**229-251.

Mimata, Y., Kamataki, A., Oikawa, S., Murakami, K., Uzuki, M., Shimamura, T. and Sawai, T. (2012). Interleukin-6 upregulates expression of ADAMTS-4 in fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Int J Rheum Dis* **15:**36-44.

Mizui, Y., Yamazaki, K., Kuboi, Y., Sagane, K. and Tanaka, I. (2000). Characterization of 5'-flanking region of human aggrecanase-1 (ADAMTS4) gene. *Mol Biol Rep* **27:**167-173.

Moon, S. K., Cha, B. Y. and Kim, C. H. (2004). ERK1/2 mediates TNF-alpha-induced matrix metalloproteinase-9 expression in human vascular smooth muscle cells via the regulation of NF-kappaB and AP-1: Involvement of the ras dependent pathway. *J Cell Physiol* **198:**417-427.

Moore, K. J. and Freeman, M. W. (2006). Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler Thromb Vasc Biol* **26:**1702-1711.

Moore, K. J., Kunjathoor, V. V., Koehn, S. L., Manning, J. J., Tseng, A. A., Silver, J. M., McKee, M. *et al.* (2005). Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis in hyperlipidemic mice. *J Clin Invest* **115:**2192-2201.

Moreno, J. A., Muñoz-García, B., Martín-Ventura, J. L., Madrigal-Matute, J., Orbe, J., Páramo, J. A., Ortega, L. *et al.* (2009). The CD163-expressing macrophages recognize and internalize TWEAK: potential consequences in atherosclerosis. *Atherosclerosis* **207:**103-110.

Morgensztern, D. and McLeod, H. L. (2005). PI3K/Akt/mTOR pathway as a target for cancer therapy. *Anticancer Drugs* **16:**797-803.

Mort, J. S., Flannery, C. R., Makkerh, J., Krupa, J. C. and Lee, E. R. (2003). Use of anti-neoepitope antibodies for the analysis of degradative events in cartilage and the molecular basis for neoepitope specificity. *Biochem Soc Symp* 107-114.

Moseley, T. A., Haudenschild, D. R., Rose, L. and Reddi, A. H. (2003). Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev* **14:**155-174.

Nagashima, H., Aoka, Y., Sakomura, Y., Uto, K., Sakuta, A., Aomi, S., Kurosawa, H. *et al.* (2004). Matrix metalloproteinase 2 is suppressed by trapidil, a CD40-CD40 ligand pathway inhibitor, in human abdominal aortic aneurysm wall. *J Vasc Surg* **39:**447-453.

Nakamura, M., Okura, T., Kitami, Y. and Hiwada, K. (2001). Nuclear factor 1 is a negative regulator of gadd153 gene expression in vascular smooth muscle cells. *Hypertension* **37:**419-424.

Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L. and Ross, R. (1994). ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb* **14:**133-140.

Nareika, A., Sundararaj, K. P., Im, Y. B., Game, B. A., Lopes-Virella, M. F. and Huang, Y. (2009). High glucose and interferon gamma synergistically stimulate MMP-1 expression in U937 macrophages by increasing transcription factor STAT1 activity. *Atherosclerosis* **202:**363-371.

Newby, A. C. (2005). Dual Role of Matrix Metalloproteinases (Matrixins) in Intimal Thickening and Atherosclerotic Plaque Rupture. *Physiological Reviews* **85:**1-31.

Newby, A. C. (2006). Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. *Cardiovascular Research* **69:**614-624.

Newby, A. C. (2007). Metalloproteinases and vulnerable atherosclerotic plaques. *Trends Cardiovasc Med* **17:**253-258.

Newby, A. C. (2008). Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability. *Atherosclerosis, Thrombosis, and Vascular Biology* **28:**2108-2114.

Newby, A. C. (2012). Matrix metalloproteinase inhibition therapy for vascular diseases. *Vascul Pharmacol*.

Newby, A. C., George, S. J., Ismail, Y., Johnson, J. L., Sala-Newby, G. B. and Thomas, A. C. (2009). Vulnerable atherosclerotic plaque metalloproteinases and foam cell phenotypes. *Thromb Haemost* **101:**1006-1011.

Newby, A. C. and Zaltsman, A. B. (1999). Fibrous cap formation or destruction - The critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovascular Research* **41:**345-360.

Nikkari, S. T., O'Brien, K. D., Ferguson, M., Hatsukami, T., Welgus, H. G., Alpers, C. E. and Clowes, A. W. (1995). Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis. *Circulation* **92:**1393-1398.

Nogueira, L., Ruiz-Ontañon, P., Vazquez-Barquero, A., Moris, F. and Fernandez-Luna, J. L. (2011). The NFκB pathway: a therapeutic target in glioblastoma. *Oncotarget* **2:**646-653.

Oram, J. F. (2000). Tangier disease and ABCA1. *Biochim Biophys Acta* **1529:**321-330.

Owens, L. V., Xu, L., Marston, W. A., Yang, X., Farber, M. A., Iacocca, M. V., Cance, W. G. *et al.* (2001). Overexpression of the focal adhesion kinase (p125FAK) in the vascular smooth muscle cells of intimal hyperplasia. *J Vasc Surg* **34:**344-349.

Paddison, P. J., Caudy, A. A. and Bernstein, E. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16:**948-958.

Paintlia, M. K., Paintlia, A. S., Singh, A. K. and Singh, I. (2011). Synergistic activity of interleukin-17 and tumor necrosis factor-α enhances oxidative stress-mediated oligodendrocyte apoptosis. *J Neurochem* **116:**508-521.

Palmer, G., Lipsky, B. P., Smithgall, M. D., Meininger, D., Siu, S., Talabot-Ayer, D., Gabay, C. *et al.* (2008). The IL-1 receptor accessory protein (AcP) is required for IL-33 signaling and soluble AcP enhances the ability of soluble ST2 to inhibit IL-33. *Cytokine* **42:**358-364.

Pamukcu, B., Lip, G. Y. and Shantsila, E. (2011). The nuclear factor--kappa B pathway in atherosclerosis: a potential therapeutic target for atherothrombotic vascular disease. *Thromb Res* **128:**117-123.

Papadakis, K. A., Prehn, J. L., Landers, C., Han, Q., Luo, X., Cha, S. C., Wei, P. *et al.* (2004). TL1A synergizes with IL-12 and IL-18 to enhance IFN-gamma production in human T cells and NK cells. *J Immunol* **172:**7002-7007.

Papadakis, K. A., Zhu, D., Prehn, J. L., Landers, C., Avanesyan, A., Lafkas, G. and Targan, S. R. (2005). Dominant role for TL1A/DR3 pathway in IL-12 plus IL-18-induced IFN-gamma production by peripheral blood and mucosal CCR9+ T lymphocytes. *J Immunol* **174:**4985-4990.

Park, E. K., Jung, H. S., Yang, H. I., Yoo, M. C., Kim, C. and Kim, K. S. (2007). Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm Res* **56:**45-50.

Park, Y. M., Febbraio, M. and Silverstein, R. L. (2009). CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *J Clin Invest* **119:**136-145.

Pauly, R. R., Passaniti, A., Bilato, C., Monticone, R., Cheng, L., Papadopoulos, N., Gluzband, Y. A. *et al.* (1994). Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. *Circ Res* **75:**41-54.

Pendse, A. A., Arbones-Mainar, J. M., Johnson, L. A., Altenburg, M. K. and Maeda, N. (2009). Apolipoprotein E knock-out and knock-in mice: atherosclerosis, metabolic syndrome, and beyond. *J Lipid Res* **50 Suppl:**S178-182.

Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J. G., Rubin, E. M. *et al.* (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* **71:**343-353.

Pobezinskaya, Y. L., Choksi, S., Morgan, M. J., Cao, X. and Liu, Z. G. (2011). The adaptor protein TRADD is essential for TNF-like ligand 1A/death receptor 3 signaling. *J Immunol* **186:**5212-5216.

Pombo-Suarez, M., Calaza, M., Gomez-Reino, J. J. and Gonzalez, A. (2008). Reference genes for normalization of gene expression studies in human osteoarthritic articular cartilage. *BMC Mol Biol* **9:**17.

Porter, S., Clark, I. M., Kevorkian, L. and Edwards, D. R. (2005). The ADAMTS metalloproteinases. *Biochem J* **386:**15-27.

Powell, A. J., Little, C. B. and Hughes, C. E. (2007). Low molecular weight isoforms of the aggrecanases are responsible for the cytokine-induced proteolysis of aggrecan in a porcine chondrocyte culture system. *Arthritis Rheum* **56:**3010-3019.

Prasadam, I., Crawford, R. and Xiao, Y. (2012). Aggravation of ADAMTS and matrix metalloproteinase production and role of ERK1/2 pathway in the interaction of osteoarthritic subchondral bone osteoblasts and articular cartilage chondrocytes -- possible pathogenic role in osteoarthritis. *J Rheumatol* **39:**621-634.

Quillard, T., Tesmenitsky, Y., Croce, K., Travers, R., Shvartz, E., Koskinas, K. C., Sukhova, G. K. *et al.* (2011). Selective inhibition of matrix metalloproteinase-13 increases collagen content of established mouse atherosclerosis. *Arterioscler Thromb Vasc Biol* **31:**2464-2472.

Rao, D. D., Senzer, N., Cleary, M. A. and Nemunaitis, J. (2009a). Comparative assessment of siRNA and shRNA off target effects: what is slowing clinical development. *Cancer Gene Ther* **16:**807-809.

Rao, D. D., Vorhies, J. S., Senzer, N. and Nemunaitis, J. (2009b). siRNA vs. shRNA: similarities and differences. *Adv Drug Deliv Rev* **61:**746-759.

Reiss, A. B., Patel, C. A., Rahman, M. M., Chan, E. S., Hasneen, K., Montesinos, M. C., Trachman, J. D. *et al.* (2004). Interferon-gamma impedes reverse cholesterol transport and promotes foam cell transformation in THP-1 human monocytes/macrophages. *Med Sci Monit* **10:**BR420-425.

Rigamonti, E., Chinetti-Gbaguidi, G. and Staels, B. (2008). Regulation of macrophage functions by PPAR-alpha, PPAR-gamma, and LXRs in mice and men. *Arterioscler Thromb Vasc Biol* **28:**1050-1059.

Rodríguez-Manzaneque, J. C., Westling, J., Thai, S. N., Luque, A., Knauper, V., Murphy, G., Sandy, J. D. *et al.* (2002). ADAMTS1 cleaves aggrecan at multiple sites and is differentially inhibited by metalloproteinase inhibitors. *Biochem Biophys Res Commun* **293:**501-508.

Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Benjamin, E. J., Berry, J. D., Borden, W. B., Bravata, D. M. *et al.* (2012). Heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation* **125:**e2-e220.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362:**801-809.

Rudijanto, A. (2007). The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. *Acta medica Indonesia* **39:**86-93.

Ruiz-Ortega, M., Rodriguez-Vita, J., Sanchez-Lopez, E., Carvajal, G. and Egido, J. (2007). TGF-β signalling in vascular fibrosis. *Cardiovascular Research* **74:**196-206.

Rutz, S. and Scheffold, A. (2004). Towards in vivo application of RNA interference - new toys, old problems. *Arthritis Res Ther* **6:**78-85.

Salter, R. C., Arnaoutakis, K., Michael, D. R., Singh, N. N., Ashlin, T. G., Buckley, M. L., Kwan, A. P. *et al.* (2011). The expression of a disintegrin and metalloproteinase with thrombospondin motifs 4 in human macrophages is inhibited by the anti-atherogenic cytokine transforming growth factor-β and requires Smads, p38 mitogen-activated protein kinase and c-Jun. *Int J Biochem Cell Biol* **43:**805-811.

Salter, R. C., Ashlin, T. G., Kwan, A. P. and Ramji, D. P. (2010). ADAMTS proteases: key roles in atherosclerosis? *J Mol Med (Berl)* **88:**1203-1211.

Sandy, J. D., Westling, J., Kenagy, R. D., Iruela-Arispe, M. L., Verscharen, C., Rodriguez-Mazaneque, J. C., Zimmermann, D. R. *et al.* (2001). Versican V1 proteolysis in human aorta in vivo occurs at the Glu441-Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4. *J Biol Chem* **276:**13372-13378.

Schaeffer, H. J. and Weber, M. J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* **19:**2435-2444.

Schindler, C., Levy, D. E. and Decker, T. (2007). JAK-STAT signaling: from interferons to cytokines. *J Biol Chem* **282:**20059-20063.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G. *et al.* (2005). IL-33, and interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23:**479-490.

Schönbeck, U., Sukhova, G. K., Shimizu, K., Mach, F. and Libby, P. (2000). Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. *Proc Natl Acad Sci U S A* **97:**7458-7463.

Shahrara, S., Pickens, S. R., Dorfleutner, A. and Pope, R. M. (2009). IL-17 induces monocyte migration in rheumatoid arthritis. *J Immunol* **182:**3884-3891.

Sharp, P. A. (2001). RNA interference--2001. *Genes Dev* **15:**485-490.

Shi, Y. (2003). Mammalian RNAi for the masses. *Trends Genet* **19:**9-12.

Shimada, K. (2009). Immune system and atherosclerotic disease: heterogeneity of leukocyte subsets participating in the pathogenesis of atherosclerosis. *Circ J* **73:**994-1001.

Shofuda, T., Shofuda, K., Ferri, N., Kenagy, R. D., Raines, E. W. and Clowes, A. W. (2004). Cleavage of focal adhesion kinase in vascular smooth muscle cells overexpressing membrane-type matrix metalloproteinases. *Arterioscler Thromb Vasc Biol* **24:**839-844.

Simon, A., Gariepy, J., Chironi, G., Megnien, J. L. and Levenson, J. (2002). Intima-media thickness: a new tool for diagnosis and treatment of cardiovascular risk. *J Hypertens* **20:**159-169.

Singh, N. N. and Ramji, D. P. (2006a). The role of transforming growth factor-beta in atherosclerosis. *Cytokine Growth Factor Rev* **17:**487-499.

Singh, N. N. and Ramji, D. P. (2006b). Transforming growth factor-beta-induced expression of the apolipoprotein E gene requires c-Jun N-terminal kinase, p38 kinase, and casein kinase 2. *Arterioscler Thromb Vasc Biol* **26:**1323-1329.

Smale, S. T. (2010). Luciferase assay. *Cold Spring Harb Protoc* **2010:**pdb.prot5421.

Smyth, D., McKay, C. M., Gulbransen, B. D., Phan, V. C., Wang, A. and McKay, D. M. (2012). Interferon-gamma signals via an ERK1/2-ARF6 pathway to promote bacterial internalization by gut epithelia. *Cell Microbiol*.

Song, R. H., Tortorella, M. D., Malfait, A. M., Alston, J. T., Yang, Z., Arner, E. C. and Griggs, D. W. (2007). Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. *Arthritis Rheum* **56:**575-585.

Sonnenberg, G. F., Nair, M. G., Kirn, T. J., Zaph, C., Fouser, L. A. and Artis, D. (2010). Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *J Exp Med* **207:**1293-1305.

Soutar, A. K. and Naoumova, R. P. (2007). Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nature clinical practise* **4:**214-225.

Stanton, H., Melrose, J., Little, C. B. and Fosang, A. J. (2011). Proteoglycan degradation by the ADAMTS family of proteinases. *Biochim Biophys Acta* **1812:**1616-1629.

Stanton, R. J., McSharry, B. P., Armstrong, M., Tomasec, P. and Wilkinson, G. W. G. (2008). Re-engineering adenovirus vector systems to enable high-throughput analyses of gene function. *Biotechniques* **45:**659-668.

Stringa, E., Knäuper, V., Murphy, G. and Gavrilovic, J. (2000). Collagen degradation and platelet-derived growth factor stimulate the migration of vascular smooth muscle cells. *J Cell Sci* **113 (Pt 11):**2055-2064.

Sylvester, J., Ahmad, R. and Zafarullah, M. (2011). Role of Sp1 transcription factor in Interleukin-1-induced ADAMTS-4 (aggrecanase-1) gene expression in human articular chondrocytes. *Rheumatol Int*.

Tabas, I. (2010). Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* **10:**36-46.

Tabas, I., Seimon, T., Timmins, J., Li, G. and Lim, W. (2009). Macrophage apoptosis in advanced atherosclerosis. *Ann N Y Acad Sci* **1173 Suppl 1:**E40-45.

Taleb, S., Romain, M., Ramkhelawon, B., Uyttenhove, C., Pasterkamp, G., Herbin, O., Esposito, B. *et al.* (2009). Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *J Exp Med* **206:**2067-2077.

Taleb, S., Tedgui, A. and Mallat, Z. (2008). Regulatory T-cell immunity and its relevance to atherosclerosis. *J Intern Med* **263:**489-499.

Taleb, S., Tedgui, A. and Mallat, Z. (2010). Interleukin-17: friend or foe in atherosclerosis? *Curr Opin Lipidol* **21:**404-408.

Tang, B. L. and Hong, W. (1999). ADAMTS: A novel family of proteases with an ADAM protease domain and thrombospondin 1 repeats. *Federation of European Biochemical Societies Letters* **445:**223-225.

Tare, N., Li, H., Morschauser, A., Cote-Sierra, J., Ju, G., Renzetti, L. and Lin, T. A. (2010). KU812 cells provide a novel in vitro model of the human IL-33/ST2L axis: functional responses and identification of signaling pathways. *Exp Cell Res* **316:**2527-2537.

Tedgui, A. and Mallat, Z. (2006). Cytokines and atherosclerosis: Pathogenic and regulatory pathways. *Physiological Reviews* **86:**515-581.

Thirunavukkarasu, K., Pei, Y., Moore, T. L., Wang, H., Yu, X., Geiser, A. G. and Chandrasekhar, S. (2006). Regulation of the human ADAMTS-4 promoter by transcription factors and cytokines. *Biochemical and Biophysical Research Communications* **345:**197-204.

Thirunavukkarasu, K., Pei, Y. and Wei, T. (2007). Characterization of the human ADAMTS-5 (aggrecanase-2) gene promoter. *Mol Biol Rep* **34:**225-231.

Tominaga, S. (1989). A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett* **258:**301-304.

Tortorella, M. D., Malfait, F., Barve, R. A., Shieh, H. S. and Malfait, A. M. (2009). A review of the ADAMTS family, pharmaceutical targets of the future. *Curr Pharm Des* **15:**2359-2374.

Tsimikas, S. and Miller, Y. I. (2011). Oxidative modification of lipoproteins: mechanisms, role in inflammation and potential clinical applications in cardiovascular disease. *Curr Pharm Des* **17:**27-37.

Turban, S., Beardmore, V. A., Carr, J. M., Sakamoto, K., Hajduch, E., Arthur, J. S. and Hundal, H. S. (2005). Insulin-stimulated glucose uptake does not require p38 mitogen-activated protein kinase in adipose tissue or skeletal muscle. *Diabetes* **54:**3161-3168.

Uglow, E. B., Slater, S., Sala-Newby, G. B., Aguilera-Garcia, C. M., Angelini, G. D., Newby, A. C. and George, S. J. (2003). Dismantling of cadherin-mediated cell-cell contacts modulates smooth muscle cell proliferation. *Circ Res* **92:**1314-1321.

Usui, F., Kimura, H., Ohshiro, T., Tatsumi, K., Kawashima, A., Nishiyama, A., Iwakura, Y. I. *et al.* (2012). Interleukin-17 deficiency reduced vascular inflammation and development of atherosclerosis in Western diet-induced apoE-deficient mice. *Biochem Biophys Res Commun*.

van Boxel-Dezaire, A. H. and Stark, G. R. (2007). Cell type-specific signaling in response to interferon-gamma. *Curr Top Microbiol Immunol* **316:**119-154.

van der Velde, A. E. (2010). Reverse cholesterol transport: from classical view to new insights. *World J Gastroenterol* **16:**5908-5915.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**.

Vera, J., Rateitschak, K., Lange, F., Kossow, C., Wolkenhauer, O. and Jaster, R. (2011). Systems biology of JAK-STAT signalling in human malignancies. *Prog Biophys Mol Biol* **106:**426-434.

von Vietinghoff, S. and Ley, K. (2010). Interleukin 17 in vascular inflammation. *Cytokine Growth Factor Rev* **21:**463-469.

Vérollet, C., Charrière, G. M., Labrousse, A., Cougoule, C., Le Cabec, V. and Maridonneau-Parini, I. (2011). Extracellular proteolysis in macrophage migration: losing grip for a breakthrough. *Eur J Immunol* **41:**2805-2813.

Wagsater, D., Bjork, H., Zhu, C., Bjorkegren, J., Valen, G., Hamsten, A. and Eriksson, P. (2008). ADAMTS-4 and -8 are inflammatory regulated enzymes expressed in macrophage-rich areas of human atherosclerotic plaques. *Atherosclerosis* **196:**514-522.

Wang, A. M., Doyle, M. V. and Mark, D. F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci U S A* **86:**9717-9721.

Wang, J., Markova, D., Anderson, D. G., Zheng, Z., Shapiro, I. M. and Risbud, M. V. (2011). TNF-α and IL-1β promote a disintegrin-like and metalloprotease with thrombospondin type I motif-5-mediated aggrecan degradation through syndecan-4 in intervertebral disc. *J Biol Chem* **286:**39738-39749.

Wang, L., Zheng, J., Bai, X., Liu, B., Liu, C., Xu, Q., Zhu, Y. *et al.* (2009). ADAMTS-7 Mediates Vascular Smooth Muscle Cell Migration and Neointima Formation in Balloon-Injured Rat Arteries. *Circulation Research* **104:**688-698.

Wang, X., Greilberger, J., Ratschek, M. and Jürgens, G. (2001). Oxidative modifications of LDL increase its binding to extracellular matrix from human aortic intima: influence of lesion development, lipoprotein lipase and calcium. *J Pathol* **195:**244-250.

Wang, Y., Singh, R., Lefkowitch, J. H., Rigoli, R. M. and Czaja, M. J. (2006). Tumor necrosis factor-induced toxic liver injury results from JNK2-dependent activation of caspase-8 and the mitochondrial death pathway. *J Biol Chem* **281:**15258-15267.

Wen, J., Zhu, H., Murakami, S., Leung, P. C. and MacCalman, C. D. (2006). Regulation of A Disintegrin And Metalloproteinase with ThromboSpondin repeats-1 expression in human endometrial stromal cells by gonadal steroids involves progestins, androgens, and estrogens. *J Clin Endocrinol Metab* **91:**4825-4835.

Westling, J., Gottschall, P. E., Thompson, V. P., Cockburn, A., Perides, G., Zimmermann, D. R. and Sandy, J. D. (2004). ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu405-Gln406 to generate glial hyaluronate binding protein. *Biochem J* **377:**787-795.

Wight, T. N. (2002). Versican: a versatile extracellular matrix proteoglycan in cell biology. *Current Opinion in Cell Biology* **14:**617-623.

Wight, T. N. and Merrilees, M. J. (2004). Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res* **94:**1158-1167.

Williams, H., Johnson, J. L., Jackson, C. L., White, S. J. and George, S. J. (2010a). MMP-7 mediates cleavage of N-cadherin and promotes smooth muscle cell apoptosis. *Cardiovasc Res* **87:**137-146.

Williams, O., Houseman, B. T., Kunkel, E. J., Aizenstein, B., Hoffman, R., Knight, Z. A. and Shokat, K. M. (2010b). Discovery of dual inhibitors of the immune cell PI3Ks p110delta and p110gamma: a prototype for new anti-inflammatory drugs. *Chem Biol* **17:**123-134.

Wilson, H. M. (2010). Macrophages heterogeneity in atherosclerosis - implications for therapy. *J Cell Mol Med* **14:**2055-2065.

Worley, J. R., Baugh, M. D., Hughes, D. A., Edwards, D. R., Hogan, A., Sampson, M. J. and Gavrilovic, J. (2003). Metalloproteinase expression in PMA-stimulated THP-1 cells. *The Journal of Biological Chemistry* **278:**51340-51346.

Wu, L., Yu, Y. L., Galiano, R. D., Roth, S. I. and Mustoe, T. A. (1997). Macrophage colony-stimulating factor accelerates wound healing and upregulates TGF-beta1 mRNA levels through tissue macrophages. *J Surg Res* **72:**162-169.

Yagami, A., Orihara, K., Morita, H., Futamura, K., Hashimoto, N., Matsumoto, K., Saito, H. *et al.* (2010). IL-33 mediates inflammatory responses in human lung tissue cells. *J Immunol* **185:**5743-5750.

Yamanishi, Y., Boyle, D. L., Clark, M., Maki, R. A., Tortorella, M. D., Arner, E. C. and Firestein, G. S. (2002). Expression and regulation of aggrecanase in arthritis: the role of TGF-beta. *J Immunol* **168:**1405-1412.

Young, M. R., Li, J. J., Rincón, M., Flavell, R. A., Sathyanarayana, B. K., Hunziker, R. and Colburn, N. (1999). Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. *Proc Natl Acad Sci U S A* **96:**9827-9832.

Yu, J. Y., DeRuiter, S. L. and Turner, D. L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* **99:**6047-6052.

Zha, Y., Chen, Y., Xu, F., Li, T., Zhao, C. and Cui, L. (2010a). ADAMTS4 level in patients with stable coronary artery disease and acute coronary syndromes. *Biomed Pharmacother* **64:**160-164.

Zha, Y., Chen, Y., Xu, F., Zhang, J., Li, T., Zhao, C. and Cui, L. (2010b). Elevated level of ADAMTS4 in plasma and peripheral monocytes from patients with acute coronary syndrome. *Clin Res Cardiol* **99:**781-786.

Zhang, Y., Wang, H., Ren, J., Tang, X., Jing, Y., Xing, D., Zhao, G. *et al.* (2012). IL-17A Synergizes with IFN-γ to Upregulate iNOS and NO Production and Inhibit Chlamydial Growth. *PLoS One* **7:**e39214.

Zhu, S. and Qian, Y. (2012). IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential. *Clin Sci (Lond)* **122:**487-511.

Zuckerman, S. H., Evans, G. F. and O'Neal, L. (1992). Cytokine regulation of macrophage apo E secretion: opposing effects of GM-CSF and TGF-beta. *Atherosclerosis* **96:**203-214.