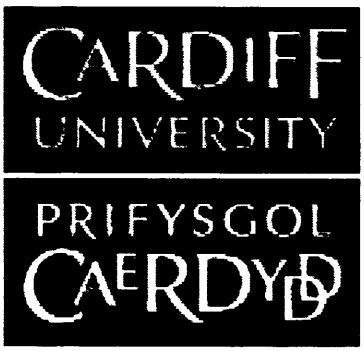
STUDIES ON INTERFERON-γ SIGNALLING AND THE REGULATION OF GENE EXPRESSION IN MACROPHAGES



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Abstract

Interferon (IFN)-γ plays a central role in the pathogenesis of atherosclerosis. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is the most widely used mechanism for IFN-γ signalling and STAT1 Ser727 phosphorylation is known to be required for its full transcriptional activity. Agonists for peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) as well as statins have been shown to exert profound anti-inflammatory actions and protective effects in atherosclerosis. However, the molecular mechanisms involved in the IFN-γ-stimulated STAT1 Ser727 phosphorylation and the anti-inflammatory actions of these therapeutic agents, particularly in relation to IFN-γ responses are yet to be fully elucidated. Studying such mechanisms may lead to the identification of new avenues for the treatment of this disease, and was therefore the main focus of studies.

Inhibitors for extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), casein kinase 2 (CK2), phosphoinostide 3'-kinase (PI3K), protein kinase C-δ (PKC-δ) and calcium-calmodulin-dependent protein kinase II (CaMKII) individually inhibited the IFN-γ-stimulated STAT1 Ser727 phosphorylation. The most extensive inhibition was observed for ERK and JNK and hence their roles in IFN-γ signalling pathway were investigated in more detail. The ERK inhibitor, PD98059 attenuated the IFN-γ-induced expression of a range of genes implicated in atherosclerosis. A role for the ERK- and JNK-cascades in the regulation of STAT1 transactivation and MCP-1 gene expression by IFN-γ was further confirmed through co-transfection assays with plasmids specifying for inactive mutant forms of Ras, Raf-1, ERK1, ERK2, MEK1 and JNK. The important roles of ERK and JNK in IFN-γ signalling were further supported by kinase assays that showed that the activity of ERK and JNK was induced approximately 3- and 1.5-fold respectively in response to IFN-γ.

RT-PCR analysis also revealed an inhibitory effect of agonists for PPARs and LXRs, as well as statins, on the IFN-γ-induced expression of several genes implicated in atherosclerosis with agent-specific actions being observed. These agents were also found to inhibit the IFN-γ-stimulated STAT1 transactivation and the activation of MCP-1 gene promoter. Endogenous ligands for LXRs, but not agonists for PPARs or statins, attenuated the IFN-γ-induced STAT1 phosphorylation, DNA binding activity and nuclear level of this transcription factor. Co-transfection assays revealed that the inhibitory effects on STAT1 activity by the LXR endogenous ligands were dependent on the corresponding receptor and that the repressive actions of agonists for PPARs and LXRs were not dependent on a mechanism involving competing for a limiting amount of shared co-activators in the cells.

These studies together reveal novel roles for ERK and JNK, via modulation of STAT1 Ser727 phosphorylation, in the regulation of gene expression by IFN-γ. In addition, the work has demonstrated the inhibitory effects of agonists for PPARs and LXRs as well as statins on the IFN-γ-induced inflammatory gene expression and potential mechanisms underlying such regulation.

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Harvey, E. J., **Li, N,** Ramji, D. P. (2007) Critical Role for Casein Kinase 2 and Phosphoinositide-3-Kinase in the Interferon-γ-Induced Expression of Monocyte Chemoattractant Protein-1 and Other Key Genes Implicated in Atherosclerosis. *Arterioscler Thromb Vasc Biol* 27 (4), pp. 806-812

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Abbreviations

Abbreviation	Full term
15d-PGJ2	15-deoxy-$\Delta^{12,14}$-PGJ_2
ABCA	ATP-binding cassette transporter A
ACAT	Acyl-CoA cholesterol acyltransferase
AP-1	Activator protein-1
APC	Antigen-presenting cells
apoE	Apolipoprotein E
APS	Ammonium persulphate
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BMM	Bone marrow-derived macrophage
bp	Basepair
BSA	Bovine serum albumin
C/EBP	CAAT-enhancer binding protein
CVD	Cardiovascular disease
CamKII	Calcium-calmodulin-dependent protein kinase II
CBP	CREB-binding protein
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Complementarity determinant
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CIITA	MHC class II transactivator
CK	Casein kinase
COX	Cyclooxygenase
CREB	cAMP-responsive element binding protein
CREM	cAMP-responsive element modulator protein
CRP	C-reactive protein
CSF	Colony stimulating factor
CXCL	CXC-chemokine ligand
Da	Dalton
DIPE	Diisoprypyl ether
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

DN	Dominant negative
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
eNOS	Endothelial NOS
ERK	Extracellular signal regulated kinase
E-selectin	Endothelial selectin
FCS	Fetal calf serum
FGF	Fibroblast growth factor
g	Gravity
g	Grams
GAPDH	Glyceraldehye-3-phosphate dehydrogenase
GAS	IFN-γ activated site
h	Hour
HAT	Histone acetyl transferase
HDL	High density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HI-FCS	Heat-inactivated FCS
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
ICER	Inducible cAMP early repressor
IFN	Interferon
IFN-γR	IFN-γ receptor
IL	Interleukin
iNOS	Inducible NOS
IP-10	Interferon-γ inducible protein -10
IRF	Interferon regulatory factor
I-TAC	Interferon-inducible T-cell alpha chemoattractant
IκB	Inhibitor of κB
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kb	Kilo bases

LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
Luc	Luciferase
LXR	Liver X receptor
LXRE	Liver X receptor response element
m	Metres
M	Molar
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MEK	MAPK kinase
MHC	Major histocompatibility complex
Mig	Monokine induced by IFN-γ
min	Minutes
MIP	Macrophage inflammatory protein
MMLV	Molony murine leukaemia virus
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NFκB	Nuclear factor κB
NOS	Nitric oxide synthase
NR	Nuclear receptor
ox-LDL	Oxidised low density lipoprotein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pen	Penicillin
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
Pol	Polymerase
PPAR	Peroxisome proliferator-activated receptors
PPRE	PPAR response element
Raf	Rous sarcoma associated factor

RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
r.p.m.	Revolutions per minute
RT	Reverse transcription
RXR	Retinoid X receptor
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
sec	Second
SFM	Serum free medium
shRNA	Small hairpin RNA
siRNA	Small-interfering RNA
SMC	Smooth muscle cells
SOCS	Suppressor of cytokine signalling
SRC-1	Steroid receptor coactivator-1
STAT	Signal transducer and activator of transcription
Strep	Streptomycin
SUMO	Small ubiquitin-related modifier
Taq	Thermos aquaticus
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEMED	N, N, N, N - tetra methyl ethylene diamine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
U	Units
UT	Untreated
UV	Ultra-violet
V	Volts
v/v	Volume/volume
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoproteins
w/v	Weight/volume
RANTES	Regulated upon activation, normal T cell expressed and secreted
NcoR	Nuclear receptor corepressor
TF	Tissue factor
TBS	Tris buffered saline

CHAPTER ONE:

GENERAL INTRODUCTION

Chapter 1: General Introduction

1.1 Introduction

Cardiovascular disease (CVD) is considered as the most common cause of premature death in the UK nowadays (Allender et al., 2008). Atherosclerosis, the principle cause of CVD, is characterised by the formation of a lipid-filled plaque within the wall of arteries and known to increasingly threaten human health worldwide (Lusis et al., 2004). There is therefore an ever increasing demand for effective therapies that combat this disease.

The development of the atherosclerotic lesion is a chronic inflammatory process orchestrated by cytokines. The study of signalling mechanisms involved in the regulation of gene expression by inflammatory cytokines, with respect to atherosclerosis, offers the opportunity to uncover potential targets for therapeutic intervention in the future.

The development of the cholesterol-lowering drugs statins, in the past ten years, has had a dramatic benefit in reducing the incidence of the disease in high-risk individuals (Grundy, 1998). Recent studies have revealed that statins can exert their atheroprotective roles through effects independent of their cholesterol-lowing action. These, so called 'pleiotropic effects', are largely dependent on their anti-inflammatory actions. In addition, the nuclear receptors peroxisome proliferators-activated receptors (PPARs) and liver X receptors (LXRs) are emerging as crucial regulators of inflammation and potential targets for therapeutic intervention of atherosclerosis. The study of molecular basis of the anti-inflammatory actions of these potential therapeutic agents could lead to the identification of novel targets for the development of new therapies to combat atherosclerosis.

The focus of this thesis is on the mechanisms by which the pro-inflammatory cytokine interferon (IFN)-γ regulates gene expression, and the molecular basis underlying the anti-inflammatory effects of agonists for PPARs and LXRs, along with statins, with respect to IFN-γ actions.

1.2 Cytokines

Cytokines are small proteins (with molecular weight ≤ 30kDa) that have pleiotropic regulatory effects on haematopoiesis and cells participating in immunity and inflammation. Cytokines exert a multitude of actions on a variety of cells, including regulation of cell proliferation, differentiation, chemotaxis and other effects associated with inflammatory responses. The expression of cytokines is generally low during normal condition but induced transiently during situation such as inflammation in response to various stimuli (e.g. cytokines or hormones) (Abbas and Lichtman, 2003; Vilcek, 2003).

Cytokines are broadly classified into families on the basis of their structural features. Families include: interleukins (IL-1 to IL-26); interferons (e.g. IFN-α, IFN-γ); chemokines (chemotactic cytokines) (CC, CXC, C, and CX3C subfamilies); tumor necrosis factors (e.g. TNF-α); transforming growth factor-β family (e.g. TGF-β1); and other growth factors [e.g. platelet-derived growth factor (PDGF)] (Table 1.1) (Vilcek, 2003).

Table 1.1 Division of cytokines into families based on structural features [adapted from Vilcek (2003)]

Family	Representative members
Interleukin-2/-4	IL-2, -4, -5, GM-CSF
Interleukin-6/-12	IL-6, -12
Interferons	IFN-α,-β,-ω,-γ,-τ
Interleukin-10	IL-10,-19,-20,-22,-24
Interleukin-17	IL-17, -25
Interleukin-1	IL-1α, -β, IL-18
Tumor necrosis factors	TNF-α, -β, CD40 ligand
Transforming growth factor-β	TGF-β1
Chemokines	CXC subfamily, CC subfamily , C subfamily, CX3C subfamily

Abbreviations: IL: Interleukin; IFN: Interferon; GM-CSF: granulocyte-macrophage colony stimulating factor; TNF: Tumor necrosis factor; TGF: Transforming growth factor

1.3 Interferon-γ

The human interferon (IFN) family is divided into type I IFNs (IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω and IFN-ν) and type II IFNs (IFN-γ) (Pestka, 2007). Type I IFNs share notable sequence homology and are synthesised by most cell types (Meager, 1998). Type II IFN, IFN-γ is molecularly distinct from the type I IFNs and functions through a separate receptor (IFN-γR). IFN-γ is produced predominantly by natural killer (NK) cells and activated T-lymphocytes but can also be produced by monocytes/macrophages, B cells and dendritic cells (Freucht et al., 2001; Schroder et al., 2004). The biological properties of IFN-γ are discussed in more detail in the following sections.

1.3.1 Structure and production of IFN-γ

Human IFN-γ (molecular mass of 17kDa) is comprised of two self-associated, non-covalent, anti-parallel α-subunits, with secondary structure consisting mostly of α-helix with no β-sheet (Pestka, 2007).

IFN-γ can be produced by an array of cells largely involved in immunity and inflammation, including CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, natural killer (NK) cells, as well as B cells, professional antigen-presenting cells (APCs) and NKT cells (Schroder et al., 2004). IFN-γ produced by NK cells and professional APCs seems to be important in early stage of defence against infection, whereas T lymphocytes secrete the majority of the IFN-γ in the adaptive immune response. The pathogen encountered macrophages evoke the secretion of chemokines that attract immune cells (e.g. NK cells) to the site of inflammation, as well as IL-12 that promotes IFN-γ synthesis in these cells. The synergistic combination of IL-12 and IL-18 produced by activated immune cells (e.g. macrophages, T cells and NK cells) further stimulate IFN-γ production. In contrast, IL-4, IL-10, transforming growth factor (TGF)-β and glucocorticoids act as inhibitory regulators of IFN-γ production (Boehm et al., 1997; Abbas and Lichtman, 2003; Schroder et al., 2004).

1.3.2 Biological significance of IFN-γ

IFN-γ was originally discovered as an antiviral agent. It is now recognised to play a far broader and complex function in aspects of immune responses. The biological

roles of IFN-γ can be grouped into four major classes: anti-viral activity, immuno-modulation, inflammation, and regulation of cellular state (Table 1.2).

IFN-γ displays antiviral activity through the inhibition of several stages of viral protein synthesis and replication, as well as a variety of immuno-modulatory and inflammatory roles. Mice deficient in IFN-γ or IFN-γR display an increased susceptibility to bacterial and viral infection (Dalton et al., 1993; Van den Broeck et al., 1995). IFN-γ exerts its activity on the immune system and inflammation mainly through a number of mechanisms including stimulation of antigen presentation by inducing the expression of Class I and II major histocompatibility complex (MHC) molecules on the surface of macrophages and T-lymphocytes; antigen processing; promoting differentiation of naive T helper (Th0) cells towards Th1 phenotype; the activation of antigen-presenting cells (APCs) (e.g. macrophages) and T-lymphocytes by inducing the production of reactive oxygen intermediates and hydrogen peroxide to enhance the killing of intracellular parasites; stimulation of cytokine production in target cells; and recruitment of leukocytes to the site of inflammation mainly through increased expression of chemokines and adhesion molecules in both leukocytes and endothelial cells (Boehm et al., 1997; Stark et al., 1998; Schroder et al., 2004).

In addition to its essential roles in immune system and inflammation, IFN-γ is also known to regulate the cellular state, mainly by influencing the rate of proliferation, differentiation and apoptosis. In general, IFN-γ inhibits cell growth by modulating the expression of certain genes linked to the cell cycle (e.g. Fas) (Dai et al., 1998), and can either induce or suppress apoptosis depending on the cell type or state of differentiation (Boehm et al., 1997; Stark et al., 1998; Schroder et al., 2004).

The important role of IFN-γ in immunity and inflammation imply that the cytokine is likely to have a role in disorders associated with a chronic inflammatory reaction. Indeed, several studies have suggested a link between the differential expression of IFN-γ with the pathology of a number of inflammatory diseases, such as rheumatoid arthritis (Canete et al., 2000), glomerulosclerosis (Kitching et al., 1999), and pancreatitis (Uehara et al., 2003). In particular, IFN-γ has been shown to play central roles in atherosclerosis (McLaren and Ramji, 2009) by regulating several processes in the pathogenesis of the disease (Harvey and Ramji, 2005) (see section 1.5 for more details).

Table 1.2 Biological roles of IFN-γ

Roles of IFN-γ	Mechanisms
Anti-viral activity	Inhibition of viral protein synthesis Inhibition of viral replication
Immunomodulation	Stimulation of antigen presentation and processing Skew differentiation of Th0 towards Th1 phenotype
Inflammation	Recruitment of leukocyte into inflammatory sites Stimulation of cytokine production
Regulation of cellular state	Inhibition of proliferation Regulation of apoptosis Activation of APCs and T-lymphocytes

1.4 Atherosclerosis

Atherosclerosis is a chronic, progressive disorder characterised by the accumulation of lipids and complex fibrous elements in the large arteries. The disorder is the primary cause of CAD, which is major cause of mortality in western societies (Ross, 1993; Lusis, 2000).

1.4.1 Lesions of atherosclerosis

The pathology of atherosclerosis can be broken down simplistically into three distinct phases: early lesion formation (fatty streaks); development of a complex lesion; and plaque rupture (Figure 1.1). The earliest recognisable lesion consists of aggregation of cholesterol-engorged macrophages (foam cells) and a varying number of T-lymphocytes within the innermost layer of the artery wall (intima) (Ross, 1995). The early lesions are usually found at sites characterised by changes in blood flow, such as branches, bifurcations and curves (Ross, 1993; Lusis, 2000). Such 'fatty streak' lesions are ubiquitous in young age and not clinically significant (Ross, 1995). This initial lesion may proceed to an expanded, intermediate (fibrofatty) lesion characterised by layers of lipid-filled macrophages and T cells that alternate with a varying number of smooth muscle cells (SMCs) surrounded by a relatively poorly developed extracellular matrix (ECM) (Ross, 1999). If the response continues, the condition could lead to the formation of a fibrous cap comprised of numerous SMCs surrounded by collagen, elastic fibres and proteoglycans, which encloses proliferating SMCs, macrophages and T cells together with a lipid-rich necrotic core (Ross, 1995; Lusis, 2000). The more advanced, complex, occlusive lesions called atheroma (also known as fibrous plaques) is covered by a fibrous cap, in which numerous SMCs characteristically occupy slit-like spaces surrounded by dense layers of connective tissue matrix, including collagen and some elastic fibers. Underneath the fibrous cap, a deeper layer of SMCs and numerous macrophages and T lymphocytes are associated with a necrotic core. Beneath the core, there may be additional layers of SMCs and connective tissue matrix (Lusis, 2000).

Although a sufficiently large lesion has been detected to block blood flow, the most important clinical complication arises when the plaque becomes fragile and ruptures. Following the rupture, blood from the lumen enters the lipid core of the plaque, where the thrombus starts to form. The thrombus is densely packed fibrin covered by an

active surface made up of platelets. Eventually total arterial occlusion due to thrombosis leads to a myocardial/cerebral infarction and stroke (Davies, 2000; Lusis et al., 2004).

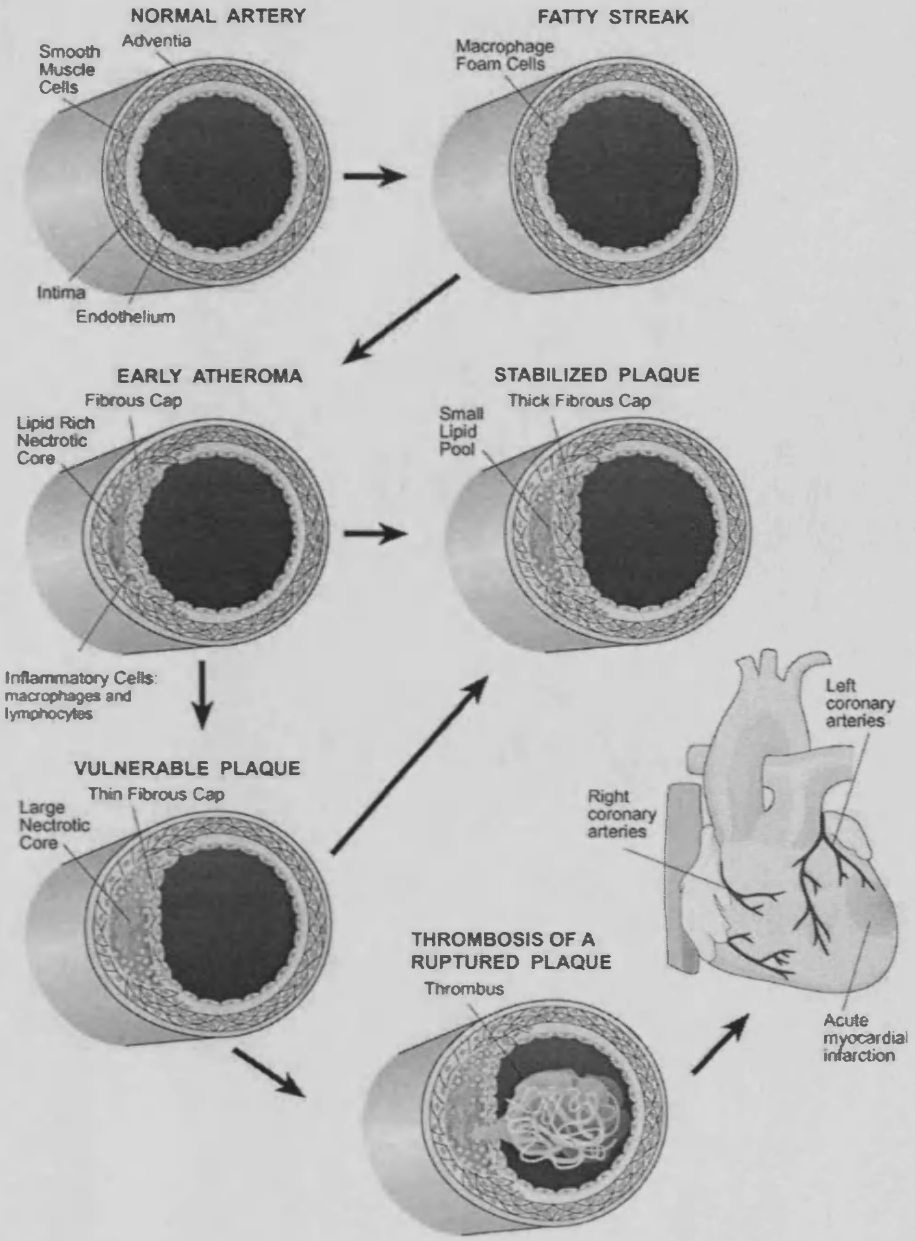


Figure 1.1 Characteristic stages of atherosclerosis lesions

The lipid-rich macrophages (foam cells) and T-lymphocytes form the earliest lesion called the fatty streak. This lesion is the precursor of more advance lesion characterised by the complex aggregation of lipid-rich necrotic debris and SMCs. Such early atheromas typically have a fibrous cap consisting of a necrotic core enclosed by SMCs and ECM. The condition develops into a more complex lesion—the fibrous plaque which could be either stable or vulnerable depending on the components. The rupture of the vulnerable plaque could initiate thrombosis, ultimately leading to acute infarction or stroke [taken from (Lusis et al., 2004)].

1.4.2 Atherosclerosis: a chronic inflammatory process

Atherosclerosis is known as a chronic inflammatory process resulting from the cellular/molecular interactions between initiators of atherosclerosis (principally lipoproteins), cellular components (monocytes/macrophages, T lymphocytes, endothelial cells and SMCs), and ECM of the artery wall (Ross, 1999; Fan and Watanabe, 2003).

1.4.2.1 Initiators of atherosclerosis

There are many risk factors considered to influence the progression of atherosclerosis, including hypercholesterolaemia, hypertension, diabetes mellitus and free radicals produced by cigarette smoking, genetic alterations, elevated plasma homocysteine concentrations and infection. From these factors, hypercholesterolaemia is regarded to be the most important risk factor as well as the principal initiator (Glass and Witztum, 2001; Steinberg, 2002). Cholesterol is carried in circulation by several lipoprotein particles including chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). In humans, LDL particles carry the majority of serum cholesterol to peripheral tissues. It has been demonstrated that LDL, VLDL and lipoproteins play a critical role in the pro-inflammatory and pro-atherogenic reactions while HDL exerts anti-inflammatory and anti-atherogenic functions (Ross, 1999; Steinberg, 2002).

When LDL particles are trapped in an arterial wall, they undergo certain modifications before they can subsequently be engulfed by macrophages. The LDL-internalised macrophages develop into foam cells contributing to fatty streak formation. Some foam cells are also derived from SMCs. Oxidation is thought to be the most important and efficient modification for the LDL being internalised by both macrophages and SMCs (Navab et al., 1996; Diaz et al., 1997; Griendling and Alexander, 1997). Cells are able to protect themselves against cholesterol-overload by down-regulating the LDL receptor (LDLR). Extensively oxidized LDL, however is no longer bound by LDLR, but rather by scavenger receptors expressed on macrophages and SMCs (Ross, 1999; Glass and Witztum, 2001).

1.4.2.2 Endothelial dysfunction

Numerous studies in humans and animals led to the response-to-injury hypothesis of atherosclerosis. The hypothesis suggests that the manifestation of the dysfunction of

the endothelium (injury) triggers the inflammatory response followed by the fibro-proliferative responses, and if unabated and excessive, the process would ultimately lead to an advanced, complicated lesion (Ross, 1993). Risk factors associated with increased atherogenesis may induce some forms of endothelial dysfunction and oxLDL is regarded to be a key factor (Ross, 1995).

Normal endothelial cells provide a non-adherent surface for leukocytes and platelets in the blood. One of the earliest atherogenic alterations seems to be the increased accumulation of lipoprotein in the ECM of the subendothelial space of the intima, probably due to increased permeability to macromolecules of endothelial cells as a result of haemodynamic forces generated by the pulsatile flow of blood (Davignon and Ganz, 2004). Indeed, the earliest lesions of atherosclerosis characteristically develop in branchpoints of artery and other regions of altered blood flow (Gimbrone et al., 2000). After being trapped into the sub-endothelial region of the intima, many of these lipoprotein particles are oxidised or glycosylated. oxLDL may induce expression of genes that seem to activate the inflammatory response (Hansson, 2001). For example, both *in vitro* and *in vivo* studies have shown that oxLDL activates the expression of vascular cell adhesion molecule-1 (VCAM-1) on the endothelium (Cybulsky and Gimbrone, 1991; Frostegard et al., 1991). VCAM-1 expression can be induced not only by pro-atherogenic molecules, but also by cytokines such as IFN-γ secreted by macrophages and T lymphocytes. In addition to VCAM-1, the expression of P-and E-selectins and other adhesion molecules are also induced through a cytokine-dependent pathway (Ross, 1995; Lusis, 2000; Hansson, 2001). Increased accumulation of lipoproteins in the sub-endothelial region results in the elevated attachment and subsequent adherence of leukocytes to the endothelial cellular surface which contributes to the recruitment of monocytes and T lymphocytes to the atherosclerotic lesion (Bonetti et al., 2003).

Following stimulation by mediators produced by a range of cells within lesions, including macrophages, platelets, SMCs and T lymphocytes, endothelial cells produce a number of growth-regulatory molecules and cytokines, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin-1 (IL-1), tumor necrosis factor α (TNFα), macrophage-colony stimulating factor (M-CSF) and monocyte chemotactic protein-1 (MCP-1) (Glass and Witztum, 2001; Hansson, 2001).

These molecules act as paracrine stimulators for neighbouring SMCs, macrophages and T lymphocytes, and hence play essential roles in lesion progression (Ross, 1995; Quehenberger, 2005; Bobryshev, 2006).

1.4.2.3 Monocyte/macrophage recruitment

Circulating monocytes attach to endothelial cells via adhesion molecules produced by endothelial cells in response to inflammatory signals, such as oxLDL. The initial adhesion involves rolling interaction followed by firmer attachment by means of selectins (L-selectin on the surface of monocytes; P-and E-selectins on the luminal surface of activated endothelium) and integrins (β1 and β2 on monocytes). The strong attachment is mediated by the interaction of the integrins with a class of ligands belonging to the immunoglobulin superfamily, most importantly intercellular adhesion molecule-1 (ICAM-1) and VCAM-1. With the low affinity for ligands in circulating monocytes, the integrins need to be activated by chemokine signals in order to mediate the firm adhesion of monocytes to the endothelium (Hansson, 2001; Quehenberger, 2005; Bobryshev, 2006). Chemokines are a type of key inflammatory cytokines that contribute to early lesion development, which not only stimulate firm adhesion, but most importantly guide the adherent monocytes and T lymphocytes across the endothelia (Burke-Gaffney et al., 2002; Braunersreuther et al., 2007a).

1.4.2.4 Specific roles of chemokines in atherosclerosis

Chemokines belong to a large superfamily of small chemotactic proteins that have been classified into four subfamilies, C, CC, CXC, and CXXXC, depending on the relative position of the first two cysteines. They are known to induce leukocyte trafficking and activation through seven transmembrane domain, G protein-coupled cell-surface receptors on target cells.

MCP-1 (also known as CCL2) is the most characterised CC chemokine which tends to attract mononuclear cells and is found at sites of chronic inflammation (Ikeda et al., 2002). It has been well established that MCP-1 plays a crucial role in inflammatory aspect of atherogenesis (Sheikine and Hansson, 2004; Zernecke and Weber, 2005). MCP-1 is produced by atheroma-associated cells (endothelial cells, SMCs, T cells and macrophages) (Boisvert, 2004). Increased expression and activity of MCP-1 and its receptor CCR2 have been identified in atherosclerotic plaques, and elevated plasma levels of MCP-1 have been found to be associated with traditional risk factors for

atherosclerosis (Ohtsuki et al., 2001; Peters and Charo, 2001; Deo et al., 2004). Activation of MCP-1/CCR-2 pathway induces the expression of adhesion molecules, pro-inflammatory cytokines and chemokines (Jiang et al., 1992; Lu et al., 1998). The overwhelming evidence for a pro-atherogenic role of MCP-1 mainly comes from studies on transgenic animals. Over-expression of MCP-1 accelerates atherosclerosis in hypercholesterolaemic animals (Aiello et al., 1999; Namiki et al., 2002). By contrast, MCP-1 or CCR-2 deficiency significantly reduces atherosclerotic lesion development in transgenic atherosclerosis-prone mice (Boring et al., 1998; Gu et al., 1998; Dawson et al., 1999; Gosling et al., 1999; Ni et al., 2001). In addition to its pro-inflammatory roles in atherosclerosis, MCP-1 has been shown to induce the expression of genes which are thought to reduce plaque stability (matrix metalloproteinases (MMP-1)) and stimulate acute thrombosis (tissue factor) (Schecter et al., 1997; Yamamoto et al., 2000). The involvement of MCP-1 in advanced lesion complication has been confirmed by studies on apolipoprotein-E (apoE)-knockout mice (Inoue et al., 2002b).

MCP-1/CCR2 is not the only chemokine/chemokine receptor involved in monocyte recruitment and key roles for other chemokines have also been documented. Macrophage inflammatory protein-1 (MIP-1) family is another structure-related pro-inflammatory CC cheomokine group, among which MIP-1α (CCL3) and MIP-1β (CCL4) are the best known to date (Maurer and von Stebut, 2004). The MIP-1 family members orchestrate acute and chronic inflammatory responses at lesion sites mainly by recruiting inflammatory cells, most crucially T-cells, but also monocytes, dentritic cells and NK cells (Menten et al., 2002). Pro-inflammatory mediators, such as TNF, IFN, lipopolysaccharide (LPS) and various infectious pathogens are known to stimulate the production of MIP in a range of atheroma-associated-cells, including monocyte/macrophages, T-cells and SMCs. A growing body of evidence suggests that MIP-1 proteins are key players in the pathogenesis of many inflammatory conditions and diseases, including asthma, wound healing, arthritis, multiple sclerosis and pneumonia (Maurer and von Stebut, 2004). Braunersreuther (2007b) and Zernecke (2006) have independently reported that deficiency of the MIP-1 receptor CCR5 reduces atherosclerotic lesion formation in atherosclerosis-prone mice. It is worth noting that CCR5 is a receptor for several chemokines, including MIP-1α, MIP-1β, MCP-2 and RANTES, all of which except MIP-1β are also ligands for CCR1

(Murdoch and Finn, 2000). Interestingly, two previous studies have reported that CCR1-/- mice do not show athero-protective immune responses as shown in CCR5-/- counterparts (Braunersreuther et al., 2007a; Zernecke et al., 2008). Therefore, it is conceivable that MIP-1β might play a pivotal role in lesion development, although direct *in vivo* and clinical evidence is required.

The IFN-γ-inducible protein (IP-10) (also known as CXCL10 as it belongs to the CXC group) is another major chemokine that is detectable in atherosclerotic lesion and its level is found to increase as lesion progresses (Heller et al., 2006). IP-10 is known to be highly induced by IFN-γ in atheroma-associated cells (i.e. macrophages, endothelial cells and SMCs). It is specifically a chemoattractant for activated T-lymphocytes, which is another key immune cellular mediator in atherosclerotic lesion progression (Luster and Ravetch, 1987). IP-10-/- and apoE-/- mice demonstrate significantly less atherosclerotic lesions compared with the apoE-/- control. Moreover, the number and activity of regulatory T-lymphocytes (T_{reg}) was significantly enhanced in lesions whereas the overall T-lymphocyte numbers were markedly diminished in IP10-deficient mice compared to wild type control (Heller et al., 2006), suggesting that this chemokine promotes atherogenesis probably not only by assisting recruitment of T cells into lesion site, but also by modulating discrete subsets of lymphocytes in the immune system towards a pro-inflammatory phenotype.

In addition to MCP-1, MIP-1 and IP-10, a continuously expanding body of chemokines have been found to play important roles in atherogenesis, such as interleukin-8 (IL-8)/CXCL8 (potent chemoattractant to macrophages), RANTES/CCL5 (towards T-cells), and fractalkine/CXC3CL1 (chemoattractant to both monocytes and T-cells) (Takahashi et al., 2002; Linton and Fazio, 2003).

1.4.2.5 Macrophages, central players in the developing plaque

Monocyte-derived macrophages, which are present at all stages of atherosclerotic lesion development, have a multifunctional role in the progression of the lesion (Jonasson et al., 1986; Weber et al., 2008). In human atherosclerotic plaques, ~40% of the cells express macrophage markers and apoE-knock out mice lacking macrophages develop very little atherosclerosis despite high cholesterol levels in the blood (Smith et al., 1995), thus demonstrating a central role for macrophages in this disease (Quehenberger, 2005; Hansson and Libby, 2006).

Macrophages express scavenger receptors, such as CD36, CD68, CXCL16, lectin-type oxidized low-density lipoprotein receptor 1 (LOX-1), scavenger receptor A (SR-A) and SR-B1, which internalize oxLDL leading to foam-cell formation, a rate-limiting step in atherosclerosis (Nicoletti et al., 1999; Quehenberger, 2005). After the uptake of oxLDL, endosomes loaded with ligand-bound scavenger receptors transfer cholesterol ester to lysosomes for intracellular degradation and also to peptide-loading compartments for MHC-class-II association. Therefore, scavenger receptors can mediate uptake of modified antigens for presentation to antigen-specific T cells and subsequently initiate the adaptive immune response (Janeway and Medzhitov, 2002). The induced expression of inflammatory cytokines, chemokines and co-stimulatory molecules in macrophages provide the additional antigen-specific stimuli and co-stimulatory molecules to activate antigen-specific T cells (Glass and Witztum, 2001; Li and Glass, 2002). Macrophages thus act as an important link between innate and adaptive immunity involved in atherogenesis.

Macrophages have two mechanisms for disposing of excess cholesterol in order to maintain cholesterol homeostasis: enzymatic modification to more soluble forms and the cholesterol efflux pathway. Cholesterol 27 hydroxylase, highly expressed in macrophages, is thought to convert cholesterol to the more soluble 27-OH-cholesterol. The expression of cholesterol 27 hydroxylase has also been observed to enhance cholesterol efflux (Escher et al., 2003). One of the principal components of the cholesterol efflux system is ATP-binding cassette transporter A1 (ABCA1) that facilitates the transport of cholesterol from cells to HDL which ultimately returns the cholesterol to the liver (Singaraja et al., 2002). ApoE can serve as an acceptor of cholesterol transported by the ABCA1-dependent pathway (Greenow et al., 2005). Due to its atheroprotective property, enhancement of this particular cholesterol efflux pathway in macrophages is thought to have important therapeutic significance (Cuchel and Rader, 2006).

1.4.2.6 Roles of macrophages in plaque destabilisation

Increased macrophage density has long been known to correlate with weakened atherosclerotic plaque cap (Lendon et al., 1991). Indeed, macrophages have now been shown to contribute to the destabilisation of the plaque in a number of ways. First, as macrophage foam cells undergo apoptosis themselves, their contents contribute to the

necrotic core (Li and Glass, 2002). Macrophages have also been shown to release the pro-thrombotic molecules, such as tissue factor (TF) and complement proteins (e.g. complement C3b) into the necrotic core to increases the thrombogenic potential of the plaque (Takahashi et al., 2002; Linton and Fazio, 2003). In addition, the proteolytic enzymes, including matrix metalloproteinases (e.g. MMP-2, MMP-9) produced by macrophages, act to weaken the fibrous cap and so lead to the eventual plaque rupture (Newby, 2008).

1.4.2.7 Cytokines in atherosclerosis

It is now well accepted that atherosclerosis is a chronic disease of the arterial wall where both innate and adaptive immuno-inflammatory mechanisms are involved. Cell types within the atheroma include monocytes/macrophages, endothelial cells, SMCs, and T-lymphocytes as well as lower numbers of dendritic cells (DCs), mast cells, a few B cells and probably natural killer T (NKT) cells. Cytokines secreted by these cells play central roles during atherosclerotic lesion development. Cytokines regulate the recruitment of inflammatory cells to the lesion through: chemotaxis; modulation of expresson of cell adhesion molecules by endothelial cells, SMCs and macrophages; and the regulation of cell proliferation and migration in the atheroma. Cytokine-induced expression of some genes, such as scavenger receptor (SR)-A and CD36, are involed in the uptake of modified lipoprotein (e.g. oxLDL), resulting in the cytoplasmic vacuoles of lipids that are the hallmark of foam cells. After proteolytic processing, fragments of the protein component of LDL bind major histocompatibility complex molecules (MHC) class II molecules and traffic to the cell surface. The specific molecular epitopes presented on professional antigen-presenting cells (APCs) (e.g. macrophages) are thought to be recognised by antigen receptors on T cells (TCR) and activate T lymphocytes and hence trigger the adaptive T cell immunity. [see (Grainger, 2004; Mehra et al., 2005; Hansson and Libby, 2006; Tedgui and Mallat, 2006; Kleemann et al., 2008; Loppnow et al., 2008; Weber et al., 2008) for reviews]. Most ox-LDL reactive CD4+T cells of murine and human atherosclerotic plaques are of the Th1 phenotype producing Th1 cytokines, such as IL-2, IFN-γ and TNF-α. Several consistent experimental studies have clearly shown a critical pro-inflammatory and pro-atherogenic role for these cytokines. On the other hand, Th2 cells, which are less abundant, compared to Th1 cells in the atheroma, produce cytokine such as IL-10, which is thought to be athero-protective due to its

anti-inflammatory properties. Indeed, the chronic vascular inflammation during atherosclerotic lesion development is thought to be probably the result of pro-inflammatory and pro-atherogenic cytokines (e.g. TNF-α, IFN-γ and chemokines) overweighing the anti-inflammatory signals (e.g. TGF-β and IL-10). Cytokines also modulate plaque stability by regulating the expression of effectors of ECM turnover, and to thrombosis through the regulation of genes involved in coagulation cascades [see (Daugherty et al., 2005; Mehra et al., 2005; Tedgui and Mallat, 2006) for reviews]. Among these cytokines, some of key players exert their crucial roles at all stages of the lesion development, such as IFN-γ.

1.5 The roles of IFN-γ in atherosclerosis

IFN-γ plays a central role in the pathogenesis of atherosclerosis. The cytokine stimulates a wide range of immune cellular responses, including the regulation of antigen presentation, control of Th1/Th2 adaptive immune response, immune cell activation and cytokine secretion (Mallat and Tedgui, 2004; Gattoni et al., 2006; Schoenborn et al., 2007). These responses, together with the IFN-γ-mediated regulation of other cellular activities, such as proliferation, differentiation and apoptosis (Stark et al., 1998), all participate in the complex nature of atherosclerosis. Although this cytokine can display anti-inflammatory properties (Muhl and Pfeilschifter, 2003), IFN-γ predominantly exerts pro-inflammatory and pro-atherogenic roles throughout disesae development, ranging from early lesion formation (foam cell), to the more mature and advanced lesion progression (atheroma), to clinical complication of atherosclerotic plaques (destablisation and thrombosis) and these actions are discussed in detail below.

1.5.1 Foam cell formation

The formation of macrophage- and SMC-originating foam cells is the pathological hallmark of the early stage of atherosclerotic lesion. Monocytes are recruited into the subendothelial layer of the intima where they differentiate into macrophages and transform into foam cells (Ross, 1993, 1995; Bobryshev, 2006), due to excessive accumulation of cholesterol as a result of an imbalance in the uptake of modified LDL into the cell and efflux of cholesterol out of it (Pennings et al., 2006). IFN-γ is known to stimulate foam cell formation by promoting immune cell recruitment to the lesion and disturbing cellular cholesterol homeostasis.

1.5.1.1 Recruitment of immune cells to the lesion

Development of atherosclerotic plaque is achieved largely through persistent recruitment of monocyte/macrophages, SMCs and T lymphocytes to the lesion sites. As discussed in section 1.4.2.3-4, a large group of chemokines and adhesion molecules orchestrate the recruitment of immune cells. IFN-γ up-regulates such recruitment by inducing the expression of these molecules. For instance, IFN-γ has been reported to induce the expression of a range of key chemokines, including MCP-1, MIP-1α and β, monokine induced by IFN-γ (MIG), IFN-inducible T cell alpha chemoattractant (I-TAC) and CXC-chemokine ligand 16 (CXCL16) (Valente et

al., 1998; Boisvert, 2004; Charo and Taubman, 2004; Wuttge et al., 2004). This cytokine also acts as an important mediator in stimulating the expression of ICAM-1 and VCAM-1 on endothelial cells, SMCs and within the atheroma (Cybulsky et al., 1993; Chung et al., 2002).

The majority of macrophages and T cells in the atherosclerotic lesion are in an activated state, which involes increased secretion of pro-inflammatory cytokines and chemokines so contributing to the inflammation and growth of the plaque. IFN-γ has been shown to stimulate the differentiation and activation of macrophages (Nathan et al., 1983). T lymphocytes are activated by recognising the fragment of LDL, as a result of intracellular processing, associated with MHC class II molecules presented on the surface of APCs. IFN-γ mediates the activation of CD4+ T-cells (Th1) through increased expression of MHC Class II molecules on the surface of a variety of cell types including ECs, macrophages and SMCs (Jonasson et al., 1985; Mach et al., 1996).

1.5.1.2 Increased cholesterol accumulation in cells

The uptake of modified LDL principally involves a family of scavenger receptors, such as SR-A and CD36 (Greaves et al., 1998). Although a few early studies showed that IFN-γ inhibits the expression of these genes and foam cell formation *in vitro* (Geng and Hansson, 1992; Nakagawa et al., 1998), an increasing volume of evidence indicates that the cytokine increases, rather than decreases, the uptake of modified LDL and the expression of scavenger receptor genes, such as SR-A and CXCL16. IFN-γ has been shown to increase the uptake of acetylated or oxidised LDL by THP-1 macrophages (Reiss et al., 2004; Wuttge et al., 2004), a widely employed cell line for the study of macrophages in relation to atherosclerosis (see Table 3.1 for a list of publications using THP-1 for investigations on atherosclerosis). Expresison of chemokine/scavenger receptor CXCL16/SR-PSOX was also found to be increased by IFN-γ in human primary monocytes, THP-1 macrophages, human aortic SMCs, as well as in murine atherosclerotic lesion (Wagsater et al., 2004; Wuttge et al., 2004). The IFN-γ-induced expression of the scavenger receptor SR-A has also been reported in early differentiated THP-1 macrophages and vascular SMCs (Li et al., 1995; Grewal et al., 2001).

As discussed in section 1.4.2.5, the cholesterol efflux pathway involves the reverse transport of cholesterol out of the cells by ABC transporter family, such as ABCA1, facilitated by apoE or apoA1 serving as cholesterol acceptors. IFN-γ decreases the cholesterol efflux from macrophages by inhibiting the expression of ABCA1 and apoE (Brand et al., 1993; Garner et al., 1997; Panousis and Zuckerman, 2000). Moreover, IFN-γ has also been shown to reduce the expression of 27-hydroxylase, which assists in the removal of cholesterol from foam cells (Reiss et al., 2001).

All these pieces of evidence indicate a role for IFN-γ in promoting foam cell formation by increasing cholesterol uptake into the cells and preventing macrophage cholesterol efflux.

1.5.2 Advanced plaque formation

As the disease progresses, the atherosclerotic lesion becomes increasingly complex and the advanced plaque includes specific features such as cellular debris, a lipid-rich necrotic core and calcification (Lusis et al., 2004). IFN-γ promotes apoptosis of macrophage foam cells by inducing the expression of several genes including TRAIL, Fas and caspase 4 and 8 (Tamura et al., 1996; Inagaki et al., 2002). IFN-γ can also prime vascular SMCs to Fas-induced apoptosis, in part by trafficking of Fas to the cell surface (Rosner et al., 2006). Accelerated apoptosis of these cells has been suggested to contribute to the accummulation of necrotic debris and plaque instability in advanced lesions (Inagaki et al., 2002). IFN-γ may contribute to calcification by up-regulating the expression of 1-α-hydroxylase, which catalyses the convertion of 25-hydroxyvitamin D to 1-α, 25-dihydroxyvitamin D metabolite (Esteban et al., 2004).

1.5.3 Plaque destabilisation

The high risk of plaque rupture and thrombosis seems to be highly associated with increased foam cell content and a thin fibrous cap (Davies, 2000). IFN-γ is thought to promote plaque destabilisation by increasing the plaque cellularity and weakening the protective cap. IFN-γ weakens the fibrous cap by inhibiting the expression of collagen genes, SMC proliferation and matrix synthesis, and meanwhile increasing the population of matrix metalloproteinases (e.g. MMP-1,2,3,9) that are thought to break down the ECMs and thereby destabilise the plaque [see (Harvey and Ramji, 2005) for

review]. In addition, the activity of tissue factor, which stimulates thrombosis following plaque rupture, is enhanced by IFN-γ (Nakagomi et al., 2000). IFN-γ therefore increases the likelihood of clinical complication of atherosclerotic lesion.

1.5.4 Lessons from murine atherosclerotic models

Important insight into the roles of IFN-γ in atherogenesis has been largely gained from studies using mouse models. Wild-type mouse strains are resistant to atherosclerosis as the circulating cholesterol is transported principally by high-density lipoproteins (HDL) that participate in the reverse cholesterol transportation pathway, towards the liver. In order to develop a mouse model with atherosclerotic lesion similar to those in humans, researchers have created knockout mice, which have pathological disturbance in lipid metabolism. The knockout mice most often used today are deficient in genes for apoE and for LDLR. These mice develop atherosclerosis spontaneously (apoE-/-) or following a high-cholesterol diet (LDLR-/-) [see (Hofker MH, 1998; Jawien et al., 2004) for reviews]. Table 1.3 summarises the findings from studies into the roles of IFN-γ on atherosclerotic lesion formation using these mouse models.

IFN-γ or IFN-γ receptor (IFN-γR)-deficient atherosclerotic-prone mice exhibit significant reduction in diet-induced atherosclerosis compared with apoE-/- or LDLR-/- control mice (Gupta et al., 1997; Whitman et al., 2002a; Buono et al., 2003). More recently, Koga et al. (2007) investigated the effect of blocking of IFN-γ function by overexpressing a soluble mutant IFN-γ receptor (sIFNγR) in apoE-/- mice and found a reduced luminal plaque area. Furthermore, administration of exogenous IFN-γ enhances atherosclerosis in apoE-deficient mice (Whitman et al., 2000). These results consistently suggest an important pro-atherogenic role for IFN-γ. IL-18 has also been shown to increase atherosclerosis in apoE-deficient mice through the release of IFN-γ (Whitman et al., 2002b).

In addition, knocking-out of IFN-γ and IFN-γR in apoE-/- mice and deficiency of IFN-γR in LDLR-/- mice resulted in reduction in cellularity, amount of T lymphocytes and MHC Class II-positive cells within the lesion (Gupta et al., 1997; Whitman et al., 2002a; Buono et al., 2003), thereby suggesting an important role for IFN-γ in immune cell recruitment, cellular proliferation and activation of the adaptive immune response

in atherosclerosis *in vivo*. Consistent with these findings, administration of exogenous IFN-γ into the apoE-null mice led to the development of lesions with increased T lymphocytes and MHC Class II-positive cells (Whitman et al., 2000). Koga et al. (2007) have suggested that blocking of IFN-γ could benefit atherosclerosis by reducing local inflammatory responses and increasing plaque stability, and therefore provide a useful therapeutic target to combat this disease and reduce the likelihood of clinical complications. The potential merits of IFN-γ-deficiency in plaque stabilisation have also been proposed by an earlier study (Gupta et al., 1997).

Despite these pieces of compelling evidence supporting pro-atherogenic roles of IFN-γ, Niwa et al. (2004) showed that IFN-γ-deficient bone marrow transplanted (BMT) LDLR-/- mice develop larger atherosclerotic lesion area than LDLR-/- mice, thus suggesting an anti-atherogenic role for this cytokine produced by bone marrow (BM)-derived cells. However, the differences in lesion size in aortic arch and abdominal aorta only occurred after 6 weeks of high-fat diet (HFD) feeding, but disappeared after 12 weeks of HFD feeding. These results suggest that the IFN-γ produced by BM-derived cells is only involved in the early phase of lesion development. Interestingly, consistent with some other studies (Gupta et al., 1997; Koga et al., 2007), these authors indicated that the enlarged atherosclerotic lesions in IFN-γ -/- (BMT) mice, which contained increased collagen deposition and decreased cellularity, were probably more stable than the lesion in IFN-γ +/+ mice. It is also worth noting that the authors agreed some limitations in their study. During BMT, the mice undergo lethal total body γ-irradiation followed by bone marrow reconstitution from donors with transgenic alterations. Schiller et al. (2001) have indicated that BMT itself can accelerate atherosclerosis and IFN-γ can be up-regulated by γ-irradiation at BMT. This could be one of the explanations why IFN-γ-/- (BMT) mice develop larger atherosclerotic lesion than IFN-γ+/+ mice. Therefore, more work will be required to elucidate the precise reason why the IFN-γ produced by BM-derived cells appeared to play a different role in atherosclerosis from the IFN-γ produced by other cells.

Table 1.3 Effects of IFN-γ on atherosclerotic lesion formation in mice

Gene deficient	Effect on atherosclerotic lesion	Roles of IFN-γ in atherosclerosis	Diet	Treatment/ Procedure	References
IFN-γR/apoE	Decreased lesion size; Decreased lipid accumulation; Decreased cellularity; Increased collagen content; Increased ApoAIV rich particles in plasma	Pro-atherogenic	High fat		(Gupta et al., 1997)
IFN-γR/LDLR	Decreased lesion size; Decreased cellularity at 8 weeks; Decreased MHC Class II-positive cells No changes in serum cholesterol	Pro-atherogenic	High fat		(Buono et al., 2003)
IFN-γ/apoE	Absence of IL-18-induced atherogenesis in IFN-γ-deficient mice	Pro-atherogenic		Daily injection of IL-18	(Whitman et al., 2002b)
IFN-γ/apoE	Decresed lesion size in males only; Decreased T lymphocytes and MHC Class II-positive cells in males only	Pro-atherogenic in male only	Normal/ High fat		(Whitman et al., 2002a)

IFN-γ (BMT)/LDLR	Increased lesion size; Decreased cellularity; Increased collagen content	IFN-γ produced by BM-derived cells anti-atherogenic, but may decrease plaque stability	High fat	Bone marrow transplant	(Niwa et al., 2004)
ApoE	Increased lesion size; Increased T lymphocytes; Increased MHC Class II-positive cells; Decreased serum cholesterol	Pro-atherogenic		Daily injection of IFN-γ	(Whitman et al., 2000)
ApoE	Decreased lesion size; Decreased lipid core; Decreased macrophages; Increased SMC and fibrotic area; Decreased expression of pro-inflammatory cytokines, chemokines, VCAM-1 and MMPs, but increased expression of pro-collagen type I in aorta; No changes in serum cholesterol	Pro-atherogenic	High fat	Gene transfer of sIFNγR by injection every 2 weeks	(Koga et al., 2007)

Abbreviations: IFN, interferon; IFN-γR, interferon-γ receptor; BMT, bone marrow transplant; apoE, apolipoprotein E; LDLR, low density lipoprotein receptor; MHC, major histocomatibility complex; IL, interleukin;

1.6 JAK-STAT pathway in IFN-γ signalling

Cytokines mediate signalling through binding to specific receptors resident at the cell surface. The association of the cytokine with its receptor generally results in the alterations in receptor conformation and initiates a specific signalling cascade leading to changes in gene transcription, translation or post-translational modifications. Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway is one of the best understood mechanisms by which cytokines transduce signals that elicit specific responses in target cells (Abbas and Lichtman, 2003). This pathway has been reported to be employed by over 50 cytokines to affect gene regulation and the IFN family is included in them (Schroder et al., 2004).

1.6.1 JAK-STAT Signalling in the IFN-γ response

IFN-γ-mediated cellular signalling requires the binding of the cytokine homodimer to the IFN-γ receptor complex. The receptor complex consists of two ligand binding chains (IFNGR1) associated with two non-ligand binding, signal transducing chains (IFNGR2) (Tau and Rothman, 1999). The receptor complex is pre-assembled on the cell surface without requiring ligand binding (Krause et al., 2006). None of the receptor chains have intrinsic kinase activity and the intracellular domain of IFNGR1 and IFNGR2 chains constitutively associate with JAK1 and JAK2 respectively (Aaronson and Horvath, 2002; Leon and Zuckerman, 2005). The interaction of STAT1 with IFNGR1 results in a conformational change of IFNGR1 and although only JAK1 is required for the two receptor chains to interact, the presence of both JAK1 and JAK2 is necessary for any ligand-dependant conformational change (Krause et al., 2006).

Figure 1.2 illustrates the JAK-STAT signalling mechanism in the IFN-γ response. Following the binding of IFN-γ to the receptors, JAK2 undergoes auto-phosphorylation and the activated JAK2 in turn phosphorylates JAK1. Phosphorylated JAK1 is then able to phosphorylate a critical tyrosine residue 440 on IFNGR1, which then provides two adjacent docking sites for Src homology (SH)2 domain of latent STAT1. The receptor-associated STAT1 dimer is phosphorylated on tyrosine residue 701 (Y701), likely through phosphorylated JAK2. Phosphorylated STAT1 homodimer dissociates from the receptor and migrates to nucleus where it binds to IFN-γ-activated site (GAS) elements (consensus sequence: TTNCNNNAA)

present in the promoter of target genes to govern the transcription of IFN-γ-regulated genes [see (Platanias and Fish, 1999; Schroder et al., 2004) for reviews]. Although IFN-γ primarily signals through the STAT1 homodimer, the other complexes such as STAT heterodimer (e.g. STAT1:STAT2) have also been found to activate transcription (Darnell et al., 1994).

Many of the genes induced during the first wave of IFN-γ-regulated transcription are transcription factors themselves, most notably interferon regulatory factor-1 (IRF-1), which is able to further drive the regulation of the next wave of transcription. IRF-1 belongs to the IRF gene family, of which IRF-1, -2 and -9 all participate in IFN-γ signalling. Heterotrimers (e.g. STAT1:STAT1:IRF-9, STAT1:STAT2:IRF-9 (known as ISGF3)) as well as IRF-1 are able to bind to IFN-stimulated response element (ISRE) to control the transcription of a spectrum of IFN-regulated genes (Platanias and Fish, 1999; Schroder et al., 2004; Leon and Zuckerman, 2005; Platanias, 2005). Additionally, IRF-1 can also bind to an unusual IRF-F site, which has overlapping sequence with ISRE consensus site. The expression of STAT1 can be induced through this pathway (Taniguchi et al., 2001). IFN-γ therefore regulates its signalling pathway via a positive feedback loop involving transcriptional activation of STAT1.

1.6.2 Activation of STAT1

Earlier studies revealed that phosphorylation at Tyr701 of STAT1 by JAKs is a crucial step for STAT1 activation as it is required for the formation of STAT complex, nuclear translocation and DNA binding (Darnell et al., 1994). It has now been well established that in addition to Tyr701 phosphorylation, the full activation of STAT1-dependent transcription involves multiple mechanisms, including STAT1 serine phosphorylation and interaction with other proteins.

1.6.2.1 STAT serine phosphorylation

It has long been recognised that phosphorylation of STAT1 at serine residue (Ser727), localised in its carboxyl-terminus is essential for full IFN-γ-dependent transcriptional activation (Wen et al., 1995). The biological significance of STAT1 Ser727 phosphorylation has been confirmed *in vivo* by the use of mice expressing a serine727-alanine mutant of STAT1 (STAT1S727A), which have increased mortality after infection along with an impaired innate immune response. In addition, the

expression of IFN-γ-induced genes was strongly reduced in macrophages expressing STAT1S727A (Varinou et al., 2003).

Despite the firmly established significance of STAT1 Ser727 phosphorylation in IFN-γ signalling, the underlying mechanisms involved in the phosphorylation at this site is yet to be fully identified. It has been shown that STAT1 serine phosphorylation occurs independently of tyrosine phosphorylation and requires JAK1/2 and tyrosine 440 STAT1 docking residue of IFNGR1 (Zhu et al., 1997; Nguyen et al., 2001). Recently, several studies have suggested a number of mechanisms involved in the IFN-γ-induced STAT1 Ser727 phosphorylation and these mechanisms appear to operate in a cell type-specific manner. Involvement of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) has been reported in human monocytic THP-1 cell line and human keratinocytes respectively (Overbergh et al., 2006). Contradictory results, however, also exist in different cell types (see Table 1.4 for a list of studies focusing on the roles of MAPK on STAT serine phosphorylation). Nguyen et al. (2001) showed that IFN-γ regulates STAT1 serine phosphorylation via phosphoinositide 3-kinase (PI3K)/Akt signalling in tumour cells and fibroblasts. A study carried out by Deb et al. (2003) demonstrated that PKCδ is activated in an IFN-γ-dependent manner and kinase activity of PKCδ mediates phosphorylation of Ser727 on STAT1 in human acute promyelocytic leukemia NB-4 cell line. Interestingly, these authors also showed that the engagement of PKCδ in IFN-γ signalling occurs downstream of PI3K and proposed a JAK—PI3K—PKCδ signalling cascade. Work in our laboratory recently revealed that casein kinase 2 (CK2) and PI3K mediate the IFN-γ-regulated expression of several key genes implicated in atherosclerosis, at least in part by, modulating STAT1 serine phosphorylation, in murine macrophages (Harvey et al., 2007).

1.6.2.2 Interaction with other transcription factors and cellular proteins

The transcriptional regulation of eukaryotic genes involves the specific and ordered interaction of a large number of proteins including transcription factors, chromosomal remodelling complexes and components of the basal transcriptional machinery. Indeed, the ability of STAT-dependent transcription is largely dependent on their interaction with other transcriptional regulators. STAT1 was shown to interact with NFκB, Sp1, USF-1, PU.1 and the glucocorticoid receptor (Brierley and Fish, 2005).

Other studies have demonstrated the association between STATs and proteins that facilitate transcription through chromatin modification. p300/CBP (cAMP-response-element binding protein (CREB)-binding protein) is one of them and it belongs to a class of co-activators, namely histone acetyltransferases (HAT). These acetylate core histones leading to changes in chromatin structure and thus improved accessibility of DNA to basal transcriptional machinery (Zhang et al., 1996). IFN-activated STAT has also been reported to interact with other proteins, including general control non-depressible 5 (GCN5), brahma-related gene-1 (BRG-1) and nMYC (Brierley and Fish, 2005; Platanias, 2005). STAT1 can also associate with minichromosome maintenance 5 (MCM5) and MCM3 which interact with RNA polymerase II and function as DNA helicases (Zhang et al., 1998).

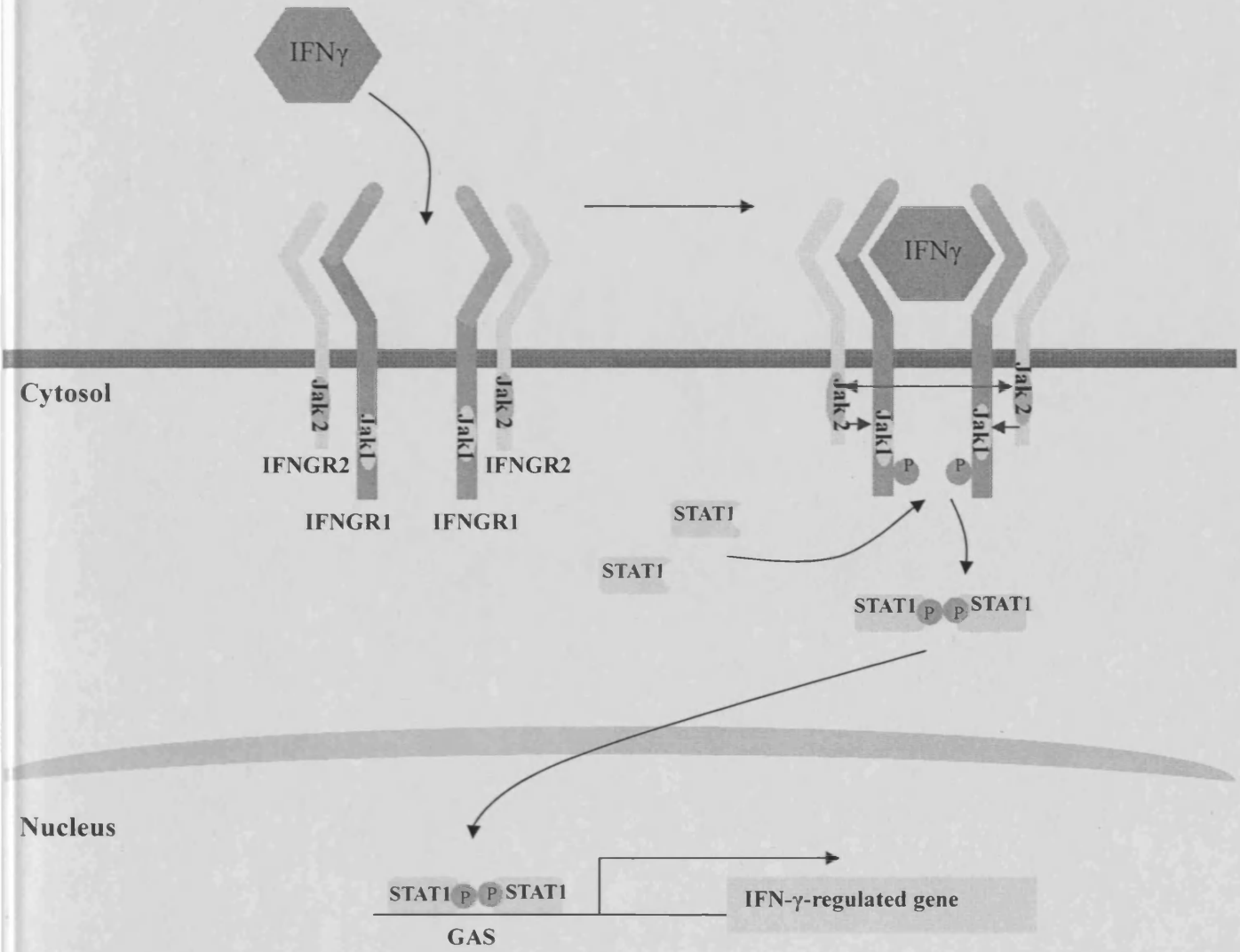


Figure 1.2 The paradigm of IFN-γ-induced JAK-STAT signalling pathway.

Inactive JAK1 and JAK2 constantly associate with IFNGR1 and IFNGR2 respectively. IFN-γ binding causes a conformation change in IFNGR, such that the inactive JAK2 undergoes auto-phosphorylation, which in turn allows JAK1 transphosphorylation by JAK2. The activated JAK1 phosphorylates tyrosine residue 440 on each IFNGR1 chain to provide docking sites for the Src homology (SH)2 domains of STAT1. Recruited STAT1 pair is phosphorylated at tyrosine residue 701. The phosphorylated STAT1 dimer dissociates from the receptor, and migrates to the nucleus where it binds to promoter IFN-γ-activation site (GAS) elements to modulate transcription of IFN-γ-regulated genes [adapted from (Schroder et al., 2004)].

1.7 Roles of mitogen-activated protein kinase (MAPK) pathways in IFN-γ signalling

1.7.1 Overview of MAPK pathways

Proper regulation of gene expression in all forms of cellular life is dependent on intracellular regulatory circuits or signal transduction pathways. Mitogen-activated protein kinase (MAPK) pathway is a key cellular signal transduction pathway that governs cellular activities ranging from gene expression, mitosis, movement, metabolism and apoptosis (Roux and Blenis, 2004) and hence has come to be appreciated as one of the most attractive targets for drug development (Hommes et al., 2003; Kaminska, 2005; O'Neill, 2006).

To date, five distinct groups of MAPKs have been characterised in mammals: extracellular signal-regulated kinases 1/2 (ERK1/2); c-Jun N-terminal kinases (JNK)/stress-activated protein kinases (SAPK) 1, 2 and 3; p38 isoforms α, β, γ and δ; ERK-3 and -4; and ERK5. The best known groups of vertebrate MAPKs are the ERK1/2, JNK/SAPK and p38 MAPK (Robinson and Cobb, 1997; Chang and Karin, 2001; Chen et al., 2001; Johnson and Lapadat, 2002). MAPKs are a three-tiered phospho-relay system composed of 3 sequentially activated kinases, a MAP kinase (MAPK), MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK). Extracellular stimuli, such as growth factors, cytokines, physical and/or chemical stress, initiate signal transduction from the cellular membrane mediated by sequential phosphorylation and activation of specific components of MAPK cascades—MAPKKK. MAPKKK-catalysed phosphorylation activates the MAPKK and induces its activity for catalysing the phosphorylation of its own substrates—MAPK (Figure 1.3). Activation of MAPK results in the phosphorylation and the subsequent activation of transcription factors, producing expression of target genes in response to particular biological stimuli. MAPKs can also catalyze the phosphorylation of several protein kinases, named MAPK-activated protein kinases (MKs) which further extend the enzymatic and amplification steps in MAPK signalling cascades [see (Chang and Karin, 2001; Pearson et al., 2001) for reviews].

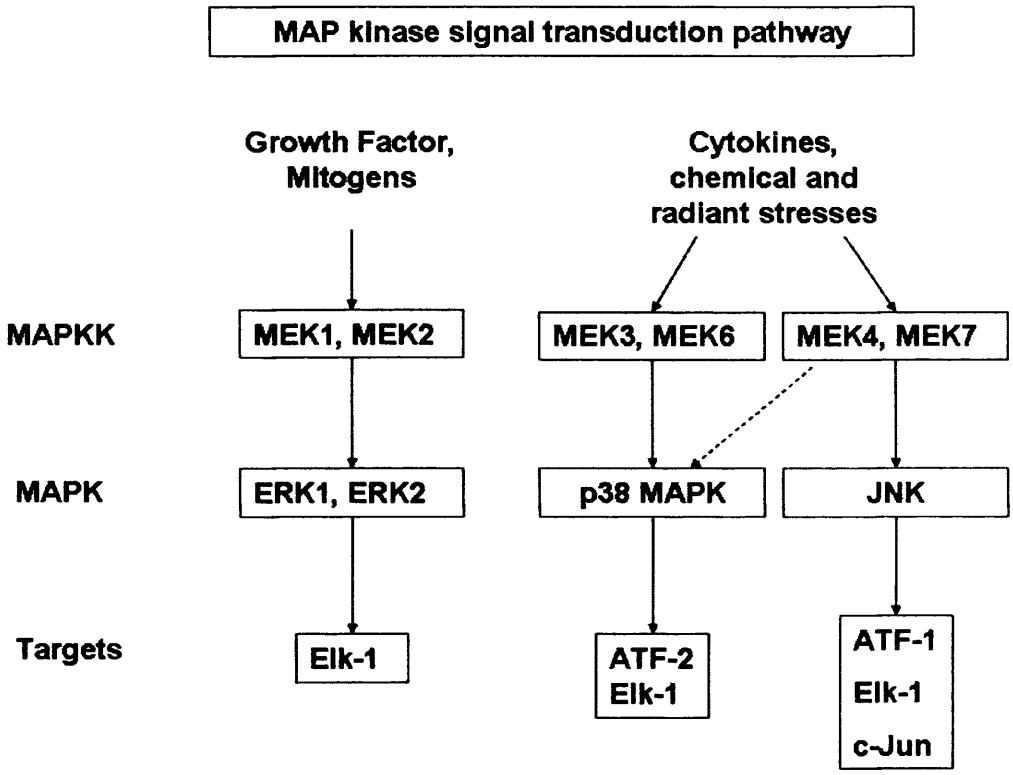


Figure 1.3 Three best known vertebrate MAP kinase pathways [adapted from (Hommes et al., 2003; Kaminska, 2005)]

The best known groups of vertebrate MAPKs are the ERK1/2, JNK/SAPK and p38. MEK1/2-ERK1/2 pathway is known to be mainly activated by growth factors and mitogens, while MEK3/6-p38 and MEK4/7-JNK/SAPK pathways are stimulated by cytokines and cellular stresses. In addition to JNK/SAPK, MEK4/7 can also act as upstream kinase of p38. Activated MAPK phosphorylate a range of transcription factors (e.g. Elk-1, ATF-2 and c-Jun), which result in the expression of target genes in response to particular biological stimuli.

Abbreviation: ATF-2: activating transcription factor-2; ERK, extracellular signal regulated kinase; JNK, c-jun-N-terminal kinase

1.7.2 Physiological and pathological roles of MAPK pathways

Collectively, numerous studies have revealed a wide range of physiological roles of MAPK pathways and different cascades are known to play distinct roles. In particular, gene targeting technology has largely contributed to our knowledge of their normal- and patho-physiological functions *in vivo*. The following sections will focus on the biological properties and functions of the best known MAPK signalling in human (ERK1/2, JNK1/2/3 and p38).

1.7.2.1 Extracellular signal-regulated kinases 1/2 (ERK1/2)

ERK signalling is known to be a vital mediator of a number of cellular fates including growth, proliferation, differentiation, survival and immune cell activation. Results from gene knockout experiments have illustrated the physiological and pathological importance of the ERK1/2 pathway. ERK2 and MEK1-knockout mice have embryonic lethality due to defective mesoderm differentiation and placenta vascularisation respectively (Giroux et al., 1999; Hatano et al., 2003). These results illustrate indispensable roles of ERK2 and MEK1 in embryonic development. In addition, ERK1 knockout mice are viable but show defective thymocyte maturation and reduced expression of α and β chains of the T cell receptor, and exhibit reduced activation by anti-CD3 antibody, illustrating that ERK1 is involved in T cell activation and inflammatory processes (Pag et al., 1999). Addition of the ERK inhibitor PD98059 has been found to block Th2 cell differentiation *in vitro*, thereby suggested that activation of the ERK pathway might play a significant role in such differentiation (Yamashita et al., 1999).

Studies via various approaches, such as pharmacological modulation, use of genetically modified animals, and expression of constitutive or dominant-negative forms of enzymes have revealed key roles of ERK activation in cardiac pathology, such as myocardial hypertrophy and ischemia/reperfusion injury, as well as in the mechanisms of cardio-protection such as ischaemic preconditioning [see (Ravingerova et al., 2003) for a review].

1.7.2.2 c-Jun N-terminal kinases (JNK)/stress-activated protein kinases (SAPK)

JNKs include JNK1 and JNK2 which are ubiquitously expressed, and JNK3, which is present primarily in the heart, brain and testis (Chang and Karin, 2001). Although individual JNK isoform-knockout mice are viable, the combined genetic disruption of

JNK1 and 2 proved embryonic lethal due to altered apoptosis program in the lateral edge of the hindbrain prior to neural tube closure, suggesting that JNK1 and JNK2 may have redundant or at least overlapping roles (Kuan et al., 1999). Moreover, as the phenotypes of these JNK-deficient animals have been further examined, it has become clear that JNK1 and JNK2 proteins in many tissues perform different cellular functions. Some of the primary evidence for the different roles for JNK1 and JNK2 came from studies for JNKs in the immune response (Dong et al., 2002). Studies on T cells in JNK1-/- and JNK2-/- mice revealed that JNK2 plays a critical role in Th cell differentiation towards Th1 and is involved directly in the production of signature cytokines of Th1 cells, such as IFN-γ, whereas JNK1 promotes Th1 differentiation via negatively regulating Th2 differentiation (Dong et al., 1998; Sabapathy et al., 1999).

Isoform-specific targeting of individual JNKs has revealed many pathological roles played by these kinases. JNK1-/- mice are protected against the following diseases: arthritis; obesity; type 2 diabetes; cardiac cell death; and non-alcoholic liver disease. Similarly, JNK2-/- mice are also protected against a broad range of pathologies: arthritis; type1 diabetes; atherosclerosis; abdominal aortic aneurysm; cardiac cell death, and TNF-induced liver damage and tumour growth (Bogoyevitch et al., 2004; Waetzig and Herdegen, 2005; Marie, 2006). In particular, knockout of the JNK2 gene in apoE-/- mice fed with high cholesterol resulted in a significant reduction in numbers of atherosclerotic plaques possibly via decrease in foam cell formation, whereas the absence of JNK1 had no effects (Ricci et al., 2004). Although the actual mechanism of JNK2 in foam cell formation remains unclear, it has been suggested that JNK2 may act through scavenger receptor A to induce apoptosis in free-cholesterol-loaded macrophages (DeVries-Seimon et al., 2005).

The expression of JNK3 is restricted in certain tissues, thereby suggesting that it plays unique physiological roles different from JNK1 and 2. In particular, studies on phenotypes of JNK3 knockout mice have revealed the significant function of JNK3 in a range of neuronal pathologies (Brecht et al., 2005). In particular, deletion of JNK3 gene has been shown to attenuate the pathological features of Parkinson's disease and Alzheimer's disease (Hunot et al., 2004).

1.7.2.3 p38 MAPK

Mammalian p38 MAPK pathway is associated with cell growth, differentiation and stress-induced apoptosis (Chang and Karin, 2001; Hommes et al., 2003). There are four p38 isoforms: p38α, p38β, p38γ, and p38δ, with p38α being the major isoform that is activated in most inflammatory cells (Dong et al., 2002). p38 MAPK has been shown to positively regulate the expression of genes coding for a range of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1β and IL-6 (Kaminska, 2005). In addition, p38 regulates the TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells, and chemoattractants such as TGF-β can induce neutrophil chemotaxis through p38 activation (Pietersma et al., 1997; Hannigan et al., 1998). Pharmacological inhibition of p38 MAPK also suggests the involvement of this kinase cascade in the immune and inflammatory responses. Fijen et al. (2001) demonstrated that inhibition of p38-MAPK by pyridinyl imidazole RWJ-67657 might be a tool to intervene in the deranged immune response in sepsis and other inflammatory diseases. Inhibition of p38-MAPK activity by SB203580 also reduced myocyte secretion of TNF-α, and prevented burn-mediated cardiac dysfunction (Ballard-Croft et al., 2001).

These observations illustrate a central role for p38 in the regulation of a wide range of immunological responses and hence, this signalling has been recognised by pharmaceutical companies as a therapeutic target for anti-inflammatory drug development (Kaminska, 2005). To date, two pharmacological inhibitors have been taken into Phase II clinical trials. SCIO-469 is in Phase II trials for pain, multiple myeloma and rheumatoid arthritis (Nikas and Drosos, 2004) whereas VX-702 has been reported to meet its primary objectives in 12-week Phase II trial in 315 patients with rheumatoid arthritis (Vertex, 2006).

1.7.3 MAPK pathways in STAT serine phosphorylation

In addition to IFN-γ as discussed in section 1.5, the significance of STAT has been well established in various signal transduction pathways induced by diverse stimuli (Decker and Kovarik, 2000). Distinct STAT isoforms are known to be employed in various stimuli-induced signalling. By and large, STAT1 is relatively specific to IFNs, STAT3 is activated by IL-6 and related cytokines, STAT4 is activated by IL-12 and IFN-α, and STAT6 is specifically activated by IL-4 and IL-13. STAT5 is activated by

various cytokines including IL-2, IL-3, erythropoietin and growth hormone (Decker and Kovarik, 2000; Yoshimura, 2006). In addition, serine phosphorylation of different STATs can be mediated through distinct signalling pathways. Three major signal transducers in MAPK pathway (i.e. ERK, JNK and p38) have all been reported to be vital in serine phosphorylation of different STAT isoforms modulated by different signals. Of note, STAT1α Ser727 is known to locate in the carboxy terminus (STAT1β lacks the region including Ser727 site) in the sequence of $PMS^{727}P$, which fits MAPK consensus recognition sequence [PXn(S/T)P (P, proline; S/T, serine/theronine; X, any amino acid; n=1 or 2)], further supporting an involvement of MAPK in STAT1 activation (Wen et al., 1995). On the other hand, there are also studies showing that the MAPK pathways are not required for STAT serine phosphorylation (see Table 1.4 for a full list of publications).

1.7.3.1 ERK

PD98059 and U0126 are both highly specific inhibitors of MEK1-ERK1/2 and MEK5-ERK5 (Davies et al., 2000), although a much higher concentration is required for inhibiting MEK5-ERK5 (Mody et al., 2001). Inhibition of phosphorylation by these inhibitors is thus thought to be a considerably specific criterion for an involvement of the ERK pathway in STAT1 and STAT3 activation in response to diverse stimuli including prolactin, IFN-γ, UV, erythropoietin and hematopoietic-specific $G\alpha_{16}$ protein (Haq et al., 2002; Lo et al., 2003; Wierenga et al., 2003; Zhang et al., 2004; Zykova et al., 2005; Kanda and Watanabe, 2007) (see Table 1.4 for the full list of studies). In addition to using pharmacological inhibitors, involvement of ERK-1 and -2 has also been demonstrated in cells where phosphorylation was shown to be either attenuated or enhanced by over-expression of dominant negative or constitutive active ERK protein respectively (Lo et al., 2003; Zykova et al., 2005). ERK2 and STAT3 have also been found to be co-immunoprecipitated from cells (Jain et al., 1998).

While these data support a role for ERKs as STAT serine kinases, several lines of evidence argue against a general validity of this concept. First, insensitivity of induced serine phosphorylation by PD98059 and U0126 in some cell lines suggests that the involvement of ERKs in the modulation of STATs serine phosphorylation is cellular- and stimuli- dependent (Gollob et al., 1999; Lim and Cao, 1999; Burysek et

al., 2002; Huang et al., 2004) (see Table 1.4 for a full list of studies). In the case of IFN-γ, only a weak and transient activation of ERK2 was observed in IFN-γ-treated mouse embryonic fibroblasts and this low level of activation did not correspond to the time course of STAT1 serine phosphorylation induced by IFN-γ (Zhu et al., 1997).

1.7.3.2 JNK/SAPK

As the ERK pathway, roles for the JNK pathway in STAT1 and STAT3 serine phosphorylation induced by various stress such as UV, anisomycin, sodium arsenite, Src oncoprotein and growth factors, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), have also been demonstrated by using specific pharmacological inhibitors (e.g. SP600125) and over-expression of dominant negative JNK proteins (Lim and Cao, 1999; Zykova et al., 2005). Absence of the response in serine phosphorylation of STAT1 and STAT3 to various signals observed in JNK1-/- and JNK2-/- cells also demonstrates the essential roles of JNK-1 and -2 in the induction of such phosphorylation (Zhang et al., 2004; Zykova et al., 2005).

Active and inactive forms of MEKK1 that phosphorylate JNK1 through JNK upstream kinase SAPK/ERK kinase 1 respectively, enhance and reduce EGF-induced STAT3 serine phosphorylation and in turn its DNA binding and transcriptional activities (Lim and Cao, 2001). The same research group has also identified that JNK1 negatively regulated the tyrosine phosphorylation and DNA binding and transcriptional activities of STAT3 stimulated by EGF (Lim and Cao, 1999). The authors thus proposed that JNK may have dual effects on STAT3 transcriptional activity, *i.e.* induction via Ser727 phosphorylation and inhibition in a Ser727-independent manner (Lim and Cao, 1999, 2001). These results indicate a critical and complex role of the MAPK pathway in the regulation of STATs.

1.7.3.3 p38 MAPK

As with investigation of the roles of ERK and JNK in STAT serine phosphorylation, specific pharmacological inhibitors, such as SB203580 and SB202190, and dominant negative plasmids have been employed in studies on the involvement of p38 MAPK in such phosphorylation. Induction of STAT1 serine phosphorylation by diverse stimuli, including UV, erythropoietin, IFNs, anisomycin and serine protease plasmin in various cell lines has been found to be sensitive to either SB203580 or SB202190 (Goh et al., 1999; Burysek et al., 2002; Haq et al., 2002; Ramsauer et al., 2002; Zhang

et al., 2004; Zykova et al., 2005; Kanda and Watanabe, 2007) (see Table 1.4 for a full list of publications). In addition, cells expressing an SB203580-resistant p38 display SB203580-insensitive, UV-induced STAT Ser727 phosphorylation, which positions p38 upstream of STAT1 in response to stress like UV (Kovarik et al., 1999).

Both STAT1 and STAT3 serine phosphorylation can be enhanced by synergistic actions such as those between IL-12 and IL-2 and such enhancement is also reduced by treatment of the cells with SB203580 (Gollob et al., 1999). p38 inhibitors have also been shown to attenuate STAT3 serine phosphorylation by serine protease plasmin (Burysek et al., 2002) and UVA (Zhang et al., 2004). p38 is also constitutively induced in src-transformed fibroblasts and functions as STAT3 kinase for serine 727 phosphorylation (Turkson et al., 1999). STAT4 is phosphorylated on serine 721 in cells transfected with a constitutively active MKK6 and p38, suggesting it too can be a target of the p38 pathway. p38 also can be activated by IL-12 and in turn phosphorylates STAT4 (Visconti et al., 2000).

Although compelling evidence for the involvement of p38 in STAT serine phosphorylation has been provided, there is controversy regarding a role of p38 in such phosphorylation in response to some stimuli, including IFN-γ. No effects of SB203580 was observed on serine phosphorylation induced by IFN-γ in macrophages (Kovarik et al., 1999) and similar level of phosphorylation was induced by IFN-γ in p38α-deficient and wild type fibroblasts (Ramsauer et al., 2002).

Table 1.4 Dependence or independence of STAT serine phosphorylation on MAPK pathways in response to various stimuli

STAT protein	Stimuli	MAPK-dependent or independent	Cell or animal used	Evidence	Reference
STAT1	Prolactin	ERK	Human neonatal foreskin keratinocytes	Sensitive to U0126	(Kanda and Watanabe, 2007)
STAT1	IFN-γ	ERK	Monocytic THP-1	Sensitive to PD98059	(Overbergh et al., 2006)
STAT1	IFN-γ	p38	Human neonatal foreskin keranocytes	Sensitive to SB203580	(Kanda and Watanabe, 2007)
STAT1	Anisomycin	p38	3T3 fibroblast from STAT1-/- mice	No phosphorylation induced by aninomycin in p38-/- cells; Reduction of phosphorylation in wild type cells by SB203580	(Ramsauer et al., 2002)
STAT1	IFN-α and -γ	p38	HeLa S3 Cells	Activation of p38 by IFN-α and –γ; Sensitive to SB203580 and dominant negative form of p38	(Goh et al., 1999)
STAT1	Synergy between LPS and IFN-γ	p38 dependent; ERK independent	Murine endothelial cells	Sensitive to SB202190; Resistant to PD98059	(Huang et al., 2004)

STAT1	Hyperosmolarity	p38 independents	African green monkey kidney fibroblasts COS-7	No effects of either over-expression of wild type or kinase deficient form of p38 and MKK6	(Bode et al., 1999)
STAT1	IFN-γ	p38 independent	3T3 fibroblasts from STAT1-/- mice and STAT1-Ser727 mutant mice; p38α-/- mouse embryonic fibroblasts	Similar level of phosphorylation observed in p38α deficient and wild type cells	(Ramsauer et al., 2002)
STAT1	UVB	ERK, JNK and p38	Mouse epidermal JB6 C141 cells; JNK1-/- and JNK2-/- cells	Sensitive to PD98059, U0126, SB203580 and SP600125; Inhibition by dominant negative forms of ERK2, JNK1 and p38; Response absent in JNK1-/- and JNK2-/- cells	(Zykova et al., 2005)
STAT1	UVA	ERK, JNK, p38	Mouse epidermal JB6 C141 cells	Sensitive to PD98059, U0126, SB202190 and PD169316; Phosphorylation blocked by dominant negative forms of p38α and JNK1; Phosphorylation blocked in JNK1/2-/- cells	(Zhang et al., 2004)
STAT1	Erythropoietin	ERK, p38	Ba/F3 cells with erythropoietin receptors	Sensitive to PD98059, U0126 and SB202190	(Haq et al., 2002)

STAT1	IFN-γ	MAPK independent	Mouse embryonic fibroblast cell line NIH 3T3	No activation of ERK, JNK or p38 by treatment with IFN-γ; No effects on STAT1 phosphorylation by dominant negative Ras; Absence of response to activated form of Ras	(Zhu et al., 1997)
STAT1/3	Serine protease plasmin	p38 dependent; ERK independent	Human primary monocytes	Sensitive to SB203580; Resistant to U0216	(Burysek et al., 2002)
STAT1/3	Synergy between IL-2 and IL-12	p38 dependent; ERK independent	Human primary T cells	Sensitive to SB203580; Resistant to PD98059	(Gollob et al., 1999)
STAT3	Erythropoietin	ERK	Ba/F3 cells with erythropoietin receptor	Sensitive to PD98059 and U0126	(Haq et al., 2002)
STAT3	Hematopoietic-specific Ga16 protein	ERK	Human embryonic kidney 293 cell line	Enhancement by over-expression of active ERK1; Sensitive to U0126; Inhibition by co-expression of dominant negative Ras and Rac-1	(Lo et al., 2003)
STAT3	UV	JNK dependent; ERK and p38 independent	African green monkey kidney fibroblast COS-1 cell line	Resistant to PD98059 and SB203580; phosphorylation by MEKK1 activated JNK1 in vitro; Phosphorylation by JNK and MEKK1 expression plasmids in vivo	(Lim and Cao, 1999)

STAT3	Epidermal growth factor/platelet derived growth factor	JNK	African green monkey kidney fibroblast COS-1 cel lline; Hela S3 cell line	Inhibition by kinase-inactive MEKK1; induced by active MEKK1	(Lim and Cao, 2001)
STAT3	Src	JNK, p38	3T3 fibroblasts	Phosphorylation by active JNK and p38	(Turkson et al., 1999)
STAT3	UVA	ERK, JNK, p38	Mouse epidermal JB6 C141 cells	Sensitive to PD98059 and SB202190; inhibition by dominant negative ERK2, JNK1 and p38; absence of phosphorylation in JNK1 -/- and JNK2-/- mice	(Zhang et al., 2004)
STAT3	Erythropoietin	ERK	Erythroid cell line ASE2	Sensitive to PD98059 and U0126	(Wierenga et al., 2003)
STAT4	IL-12	p38	Human primary lymphocytes	Activation of p38 by IL-12; phosphorylation by active p38α and MKK6	(Visconti et al., 2000)

Abbreviations: ERK, extracellular signal-regulated kinase; IL, interleukin; IFN, interferon; JNK, c-Jun N-terminal kinases

1.8 Peroxisome proliferator-activated receptors (PPAR) and liver X receptors (LXR) agonists target inflammation

Perioxisome proliferators-activated receptors (PPARs) and liver X receptors (LXRs) belong to class of nuclear receptors (NRs), so called 'adopted orphan nuclear receptors'. This class of NRs is a superfamily of ligand-activated transcription factors that regulate diverse aspects of development, immunity, homeostasis and metabolic processes (Tobin and Freedman, 2006). The most striking property of this class is its ability to form heterodimers with retinoid X receptor (RXR) to bind to DNA (Giguere, 1999). PPARs and LXRs are emerging as key regulators of inflammation, and potentially the major targets for therapeutic intervention of atherosclerosis (Marx et al., 2004; Barish, 2006; Li and Palinski, 2006; Rizzo and Fiorucci, 2006). However, the molecular mechanisms underlying the anti-inflammatory actions of PPARs and LXRs remain poorly understood (Kota, 2005). Thus, understanding the mechanisms involved in inflammatory responses could potentially benefit the development of new therapeutic approaches for intervention of atherosclerosis.

1.8.1 PPARs

1.8.1.1 Biology of PPARs

The PPAR family is composed of 3 receptor isoforms, PPARα, PPARγ and PPARδ (also known as PPARβ). All of them can form heterodimer with RXR and activate transcription by binding to a specific DNA element termed the PPAR response element (PPRE). In the absence of ligands, PPAR-RXR heterodimers can recruit corepressor complex to actively repress transcription. Upon activation by ligands, the heterodimer complex undergoes conformational changes, resulting in the release of the corepressor complex, the association of coactivators and activation of transcription of target genes (Kota, 2005). This activity enables PPARs to positively regulate gene networks involved in the control of lipid metabolism and glucose homeostasis in several tissues including adipose tissue, muscle and liver, ultimately influencing circulating lipid and glucose levels (Delerive et al., 2001; Kersten, 2002). In addition, PPARs also act negatively to regulate the expression of pro-inflammatory genes in a ligand-dependent manner and several distinct mechanisms have been proposed to date (Ricote and Glass, 2007). The details of these mechanisms are discussed below.

There are a variety of potential endogenous ligands for the PPARs (Table 1.5), including unsaturated fatty acids and naturally occurring fatty acids derivatives. Synthetic, high-affinity, selective ligands for each type of PPARs are also available, such as fibrates (PPARα agonist used to treat hypertriacylglycerolaemia) and thiazolidinediones (TZD, also known as glitazones, PPARγ agonist used to treat type 2 diabetes mellitus) (Straus and Glass, 2007).

Table 1.5 Endogenous and synthetic ligands for PPAR isoforms [adapted from (Bensinger and Tontonoz, 2008)]

PPAR isoform	Endogenous ligands	Synthetic ligands
PPARα	Unsaturated fatty acids Saturated fatty acids Leukotriene B4 8-HETE	**Fibrates:** Fenofibrate Clofibrate Gemfibrozil GW7647 Wy14643
PPARδ	Unsaturated fatty acids Saturated fatty acids Carbaprostacyclin	GW501516 L-165041
PPARγ	Unsaturated fatty acids 15-deoxy-Δ12,14-PGJ2 15-HETE 9- and 13- HODE	**TZD (glitazones):** Rosiglitazone (BRL49653) Pioglitazone Troglitazone Ciglitazone **Tyrosine derivatives:** Farglitazar GW7845

1.8.1.2 PPARs in inflammation and atherosclerosis

Substantial evidence suggests that PPARs are important regulators of the immune system and numerous studies have shown that PPAR ligands can exert anti-inflammatory roles in a range of tissues, such as lungs, joints, nervous systems and gastrointestinal tract (Bensinger and Tontonoz, 2008). The expression of all three PPAR isoforms has been observed in vascular wall cells, immune cells and atherosclerotic lesions (Neve et al., 2000; Fernandez, 2008). A growing body of evidence suggests the atheroprotective effect of PPARs and the most compelling

evidence arises from studies on transgenic mice (Table 1.6) and clinical trails. Two research groups have shown that administration of PPARα agonists fenofibrate (Duez et al., 2002) and GW7647 (Li et al., 2004) could ameliorate atherosclerotic lesion progression in LDLR- and apoE-deficient mice respectively. In addition, a number of PPARγ agonists, such as rosiglitazone (Li et al., 2004), troglitazone (Collins et al., 2001; Chen et al., 2001b), as well as PPAR-α and -γ agonist LY465608 (Zuckerman et al., 2002) have been shown to reduce the atherosclerotic lesion size in LDLR- and apoE-deficient mice. These results clearly demonstrate the anti-atherogenic effects of PPAR-α and -γ in transgenic atherosclerosis-prone mice, thereby suggesting the therapeutic potential of PPAR-α and -γ agonists in the treatment of atherosclerosis. To date, a number of clinical trails have produced promising results on the potential therapeutic benefits of PPAR-α and -γ agonists for the treatment of atherosclerosis. Fibrates have been shown to reduce the progression of coronary atherosclerosis in a number of independent clinical trails (Ericsson et al., 1997; Frick et al., 1997; Diabetes Atherosclerosis Intervention Study Investigators 2001). In addition, various studies have demonstrated potential therapeutic benefits of PPARγ agonists in restoring cardiovascular function by focusing on surrogate markers of atherosclerosis. For example, treatment of patients with coronary artery disease and type 2 diabetes mellitus with glitazones demonstrated a significant reduction in the circulating level of serum amyloid A, TNF-α and MMP9 (Marx et al., 2003a; Marx et al., 2003b). Serum amyloid A, TNF-α and MMP9 are all known to predict the risk for the development of atherosclerosis (Libby et al., 2002). Moreover, in a clinical study of 135 Japanese patients with diabetes, troglitazone treatment reduced intimal and medial complex thickening in carotid arteries, suggesting that the drug influences structural changes in arteriosclerosis (Minamikawa et al., 1998).

In contrast to the compelling evidence on the anti-inflammatory and atheroprotective effects of PPAR-α and -γ agonists, the role of PPARδ in the modulation of inflammation is less understood (Bensinger and Tontonoz, 2008) and studies on mouse models of atherosclerosis have yielded conflicting results. In LDLR-null male mice, the PPARδ agonist GW0742 reduced the expression of inflammatory genes but failed to inhibit lesion formation (Li et al., 2004). However, another group has shown that the same ligand at a similar concentration has a potent anti-atherogenic effect in LDLR-/- female mice (Graham et al., 2005). Additional study in LDLR-/- mice

transplanted with PPARδ-deficient bone marrow has suggested a pro-atherogenic effect of PPARδ (Lee et al., 2003). The authors proposed that in the absence of the ligand, PPARδ binds and sequesters Bcl-6, a repressor of atherogenic inflammation and by knocking out of PPARδ, Bcl-6 is released and represses inflammation.

These studies (Table 1.6) in mouse models of atherosclerosis have also revealed that the atheroprotective effect of PPAR ligands are significantly attributed to their anti-inflammatory actions in atherosclerotic lesion cells, most importantly, macrophages. Expression of pro-inflammatory molecules, including IFN-γ, TNF-α and chemokines (e.g. MCP-1, RANTES), have been shown to be inhibited by agonists of all three PPAR isoforms in lesions (Li et al., 2004; Graham et al., 2005; Takata et al., 2008). In addition, inhibited expression of ICAM-1 and VCAM-1 suggests that these ligands negatively regulate atherosclerotic progression, possibly at least in part, through inhibition of leukocytes adhesion to vascular endothelial cells (Li et al., 2004; Graham et al., 2005). This notion has been confirmed by a number of studies *in vitro* (Marx et al., 1999; Collins et al., 2001; Barish et al., 2008).

Therefore, investigating the molecular mechanisms of PPAR agonists in atherosclerotic lesion cells not only could improve our understanding of roles of these nuclear receptors in the development of atherosclerosis, but might also facilitate our search for novel therapies to combat this disease.

Table 1.6 Roles of PPARs on atherosclerotic lesion formation in mice

Nuclear receptor	Ligands (dose) or other treatment	Effects on atherosclerotic lesion	Role of PPAR in atherosclerosis	References	Animal	Diet
PPARα	GW7647 (2.5mg/kg/day)	Decreased lesion area; Decreased expression of IFN-γ,TNF-α, MCP-1, ICAM-1 and VCAM-1 in lesion; Decreased lipid accumulation in peritoneal macrophages	anti-atherogenic	(Li et al., 2004)	LDLR-/- male mice	high fat
PPARα	fenofibrate (100mg/kg/day)	Decreased lesion area in human apoA I transgenic mice; Decreased expression of MCP-1 mRNA in aorta	anti-atherogenic	(Duez et al., 2002)	apoE-/- mice	high fat
PPARγ	rosiglitazone (10mg/kg/day)	Decreased expression of IFN-γ, TNF-α, MCP-1, ICAM-1 and VCAM-1 in lesion; Decreased lipid accumulation in peritoneal macrophages;	anti-atherogenic in male mice	(Li et al., 2004)	LDLR-/- male mice	high fat
PPARγ	troglitazone (4g/kg/day)	Decreased lesion size; Decreased macrophage accumulation in intimal xanthomas;	anti-atherogenic	(Collins et al., 2001)	LDLR -/- Mice	high fat or high fructose
PPARγ	troglitazone (0.1% of food w/w)	Decreased lesion area; Increased HDL cholesterol level	anti-atherogenic	(Chen et al., 2001b)	apoE-/- mice	high fat

PPARγ	rosiglitazone GW7845 (20mg/kg/day)	Decreased lesion size in male mice; Decreased expression of TNF-α and gelatinase B in aortic root; No change in lesion size in female mice;	anti-atherogenic in male mice	(Li et al., 2000)	LDLR-/- mice	high fat
PPARα, γ	LY465608 (10mg/kg/day)	Decreased lesion size	anti-atherogenic	(Zuckerman et al., 2002)	apoE -/- mice	/
PPARδ	GW0742 (5mg/kg/day)	Decreased expression of IFN-γ, TNF-α, MCP-1, ICAM-1 and VCAM-1 in lesion; No change in lesion size; No change in weight and insulin level ; No change in lipid accumulation in peritoneal macrophages	anti-inflammatory in male mice	(Li et al., 2004)	LDLR-/- male mice	high fat
PPARδ	GW0742 (6 and 60mg/kg/day)	Decreased lesion area; Decreased expression of MCP-1 and ICAM-1 (lesion), TNF-α (macrophages and adipose tissue), MCP-1, RANTES, IL-12 and sTNFR1 (serum)	anti-atherogenic in female mice	(Graham et al., 2005)	LDLR-/- female mice	high fat
PPARδ	GW501516 (2mg/kg/day)	Decreased lesion area; Increased HDL cholsterol level; Decreased monocyte transmigration through an endothelial cell monolayer mediated by MCP-1 gradient	anti-atherogenic in male mice	(Barish et al., 2008)	apoE-/- male mice	high fat

PPARδ	GW0742 (1 and 10mg/kg/day)	Decreased lesion area; Decreased macrophage content in lesion; Decreased vascular expression of TNF-α, IL-6, MCP-1, CCR2, VCAM-1 and osteopontin; Increased expression of angiotensin II-inhibited Bcl-6 in aorta and peritoneal macrophages; abolished angiotensin II-stimulated phosphorylation of ERK1/2 and p38 in peritoneal macrophages	anti-atherogenic	(Takata et al., 2008)	LDLR-/- mice stimulated with angiotensin II	high fat
PPARδ	bone marrow transplant from PPARδ-/- mice	Decreased lesion size	pro-atherogenic	(Lee et al., 2003)	LDLR-/- Mice	high fat

Abbreviations: PPAR, perixisome proliferators-activated receptors; IFN, interferon; TNF, tumor necrosis factor; MCP, Monocyte chemoattractant protein; ICAM, Inter cellular adhesion molecule 1; VCAM, Vascular cell adhesion molecule; IL, interleukin; CCR, CC chemokine receptor; ERK, extracellular signal-regulated kinase; HDL, high density lipoprotein

1.8.1.3 Anti-inflammatory actions of PPAR ligands in cells associated with atherosclerosis

PPARs have been demonstrated to be expressed in cells within human and murine atherosclerotic lesions, co-localising with macrophages, endothelial cells and SMCs (Marx et al., 1998; Ricote et al., 1998; Chinetti et al., 2000a; Chinetti et al., 2000b). Several studies have indicated that PPAR ligands may exert their anti-inflammatory and protective roles in atherosclerotic lesion development by modulating immune and inflammatory responses in these cells (Figure 1.4).

Early atherogenesis is characterised by the recruitment and the subsequent entry of monocytes/macrophages to an activated endothelium and ultimately their trans-migration into the intima. This process is largely mediated by interactions between different molecules, including pro-inflammatory cytokines (e.g. IFN-γ, TNF-α), chemokines (e.g. MCP-1, IP-10), adhesion molecules (e.g. ICAM-1, VCAM-1), selectins (e.g. E-selectin) and intergrin [see (Quehenberger, 2005) for review]. Expression of chemokines is induced by various pro-inflammatory stimuli, such as IFN-γ and LPS, and PPAR-α, -γ and -δ ligands have been shown to repress the expression of these molecules in macrophages and endothelial cells (Marx et al., 2000; Pasceri et al., 2001; Lee et al., 2003; Welch et al., 2003; Kim and Kim, 2006; Barish et al., 2008; Kitajima et al., 2009). In addition, several studies have demonstrated that the expression of VCAM-1 in endothelial cells induced by TNF-α is inhibited by agonists of PPAR-γ (MCC-555) and -α agonists (fenofibrate and WY14643) (Marx et al., 1999; Xu et al., 2001; Kurebayashi et al., 2005). The anti-inflammatory effect of PPAR ligands is further confirmed by the findings that the expression of key pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1 and -8, as well as the cytokines involved in immuo-modulation such as IL-2 and -4 is suppressed by various types of ligands for all three PPAR isoforms in macrophages and SMCs (Jiang et al., 1998; Takano et al., 2000; Ryoo et al., 2004; Ding et al., 2006; Piraino et al., 2006). Repression of IFN-γ expression by fenofibrate has also been confirmed in IL-10-deficient mice (Lee et al., 2007a).

T lymphocytes and SMCs are recognised as crucial players in atherosclerotic lesion formation and complication (Hansson, 2001). After the recruitment into the vessel wall, T cells undergo differentiation into CD4+ Th1 cells after stimulation by antigens such as oxLDL. Th1 cell predominantly secrete pro-inflammatory cytokines such as

IFN-γ, TNF-α and IL-12, which can in turn activate other cellular participants in atherosclerosis (Binder et al., 2002). PPAR-α and -γ activation reduces the T cell production of IFN-γ, TNF-α, IL-2 and IL-4, thus possibly modulating multiple levels of distal responses to these cytokines during atherogenesis (Jones et al., 2002; Marx et al., 2002). In SMCs, PPARα agonists reduce IL-1- or PMA-induced cyclooxygenase 2 (COX2) expression (Staels et al., 1998). Cytokine induced COX2 expression in macrophages is also repressed by ligands for PPAR-γ (Castrillo et al., 2000; Inoue et al., 2000; Straus et al., 2000) and -δ (Welch et al., 2003). At sites of inflammation, COX promotes increased production of various inflammatory mediators (Linton and Fazio, 2002; Burleigh et al., 2005) and has been shown to promote early atherosclerotic lesion formation in apoE-null mice (Burleigh et al., 2005).

In summary, PPAR ligands exert their atheroprotective roles, at least in part by antagonising inflammatory responses within key cells involved in atherogenesis. Such anti-inflammatory effects of these ligands are mediated by inhibition of expression of inflammatory molecules, such as pro-inflammatory cytokines, chemokines and adhesion molecules. The exact molecular mechanisms underlying such repression are yet to be fully understood although a number of models have been proposed (details discussed in section below).

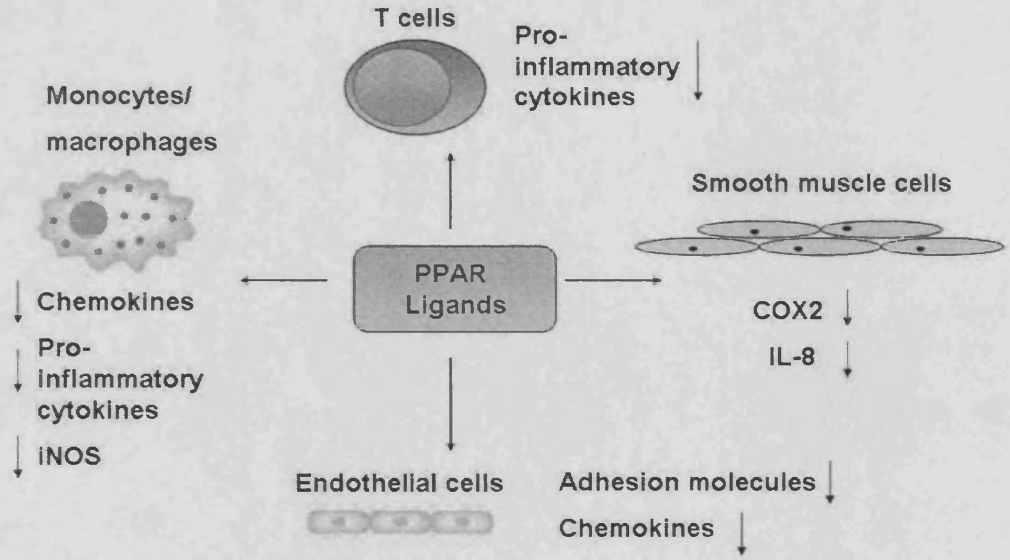


Figure 1.4 Cellular targets for the anti-inflammatory actions of PPARs.

PPAR are activated by various ligands and down-regulate diverse components of inflammatory responses such as cytokine and chemokine secretion within key atherosclerotic lesion cells

Abbreviations: PPAR, peroxisome proliferators-activated receptors; iNOS, inducible nitric oxide synthase; COX, cyclooxygenerase; IL, interleukin

1.8.1.4 Transrepression of IFN-γ signalling by PPARs

Ligand-dependent inhibition of inflammatory gene expression represents one of the key repressive functions of PPARs. This process is termed transrepression, in which PPARs interfere with signal-dependent activation of inflammatory response genes through protein-protein interactions with co-regulatory proteins and promoter-bound transcription factors, rather than direct, sequence-specific interaction with DNA (Pascual and Glass, 2006). IFN-γ is one of the key pro-inflammatory cytokines involved in inflammatory regulation and the pathogenesis of atherosclerosis (see section 1.5). An ever growing volume of evidence indicates that the potent anti-inflammatory and atheroprotective actions of PPAR ligands are mediated in part through the inhibition of the deleterious actions of IFN-γ (see Table 1.7 for a full list of publications). Of particular importance, various types of PPARγ activators inhibit the IFN-γ-induced expression of inflammatory cytokines and chemokines in a large range of cell types, such as macrophages and endothelial cells (Maggi et al., 2000; Marx et al., 2000; Park et al., 2003; Welch et al., 2003; Giri et al., 2004; Antonelli et al., 2006; Barish et al., 2008; Panzer et al., 2008).

A number of distinct molecular mechanisms underlying transrepression by PPAR ligands in IFN-γ signalling have been proposed and all of mechanisms discovered to date operate at the transcriptional activation of target genes (Ricote and Glass, 2007). First, the endogenous PPARγ ligand 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ2) antagonises the IFN-γ-induced expression of chemokines (e.g. IP-10 and MIG) and iNOS by targeting the JAK-STAT pathway (Chen et al., 2003; Weber et al., 2003, 2004; Panzer et al., 2008). Chen et al. (2003) demonstrated that 15d-PGJ2 inhibited the IFN-γ-induced expression of iNOS in the mouse macrophage RAW264.7 cell line by attenuating the phosphorylation of JAK2 and STAT1 (tyrosine 701), as well as STAT1 DNA binding activity. This agonist has also been demonstrated to inhibit the IFN-γ-mediated expression of genes, such as chemokines (e.g. IP-10, Mig and I-TAC) by similar mechanisms in mouse mesangial and insulinoma cells (Weber et al., 2003; Panzer et al., 2008). In addition, these authors indicated that 15d-PGJ2 could mediate the inhibition via targeting the IFN-γ-induced STAT1 nuclear translocation. These results together suggest that the JAK-STAT pathway may be a general target for 15d-PGJ2 in attenuating the IFN-γ-mediated gene expression. Interestingly, these research groups independently reported that 15d-PGJ2 targets IFN-γ signalling in a

PPARγ-independent manner as the PPARγ inhibitor (GW9662 or BADGE) failed to influence the effect of 15d-PGJ2 in IFN-γ-mediated signalling (Chen et al., 2003; Weber et al., 2004; Panzer et al., 2008). In addition to the endogenous PPARγ ligand, various types of the synthetic agonists (glitazone) have been shown to attenuate the IFN-γ-induced STAT1 transactivation in mouse macrophages (Ricote et al., 1998; Chen et al., 2003). However, Panzer et al. (2008) have shown that the PPARγ agonists, ciglitazone and GW9662 do not target IFN-γ-dependent JAK-STAT pathway and downstream genes in mouse mesangial cells. This suggests that the mechanism may operate in a cell type- and ligand-specific manner.

In contrast to the mechanisms detailed above, Park et al. (2003) have suggested that 15d-PGJ2 and rosiglitazone inhibit the IFN-γ-mediated expression of MCP-1, IP-10, TNF-α, IL-1β and IRF-1 by increasing the expression of suppressor of cytokine signalling (SOCS)-1 and -3, which are thought to suppress the JAK-STAT pathway via a negative feedback loop (Krebs and Hilton, 2001).

Another important mechanism by which PPAR ligands repress the cytokine-mediated expression of inflammatory response genes is to target the nuclear factor-κB (NFκB) pathway (Ricote et al., 1998; Straus et al., 2000; Takano et al., 2000; Xu et al., 2001; Ding et al., 2006; Piraino et al., 2006). NF-κB is a dimer composed of various subunits that are members of the Rel family of proteins [p105/50, p100/52, p65 (RelA), RelB and c-Rel]. In resting cells, NF-κB proteins reside in the cytoplasm where they are sequestered by a family of inhibitors of κB (IκB) proteins. Upon stimulation with pro-inflammatory mediators including cytokines, the activated NF-κB complex translocate to the nucleus. This activation of NFκB requires phosphorylation by IκB kinase (IKK) of IκB protein at specific serine residues, which target these proteins for ubiquitin conjugation and degradation by the 26S proteasome. Activation of IKK is mediated by phosphorylation through various upstream kinases, such as NFκB-inducing kinases, Akt and PKCζ [see (Ghosh and Karin, 2002) for review]. 15d-PGJ2 has been shown to suppress the IFN-γ/LPS-synergistically mediated gene expression, including pro-inflammatory cytokines and COX2, by inhibiting the activity of IKK, resulting in impaired degradation of IκB (Castrillo et al., 2000), and this further leads to the inhibition of p65 (NFκB) nuclear translocation and recruitment of the transcriptional co-activator p300 by p65 (Giri et al., 2004). Giri et

al. (2004) also indicated that 15d-PGJ2 down-regulates the IFN-γ/LPS-mediated PI3K-Akt pathway. In addition to 15d-PGJ2, synthetic PPARγ agonists, such as rosiglitazone and troglitazone also attenuate the IFN-γ-induced gene expression (e.g. chemokines and iNOS) by down-regulating NFκB DNA binding activity (Marx et al., 2000) and transcriptional activity (Ricote et al., 1998).

Overall, these studies suggest that PPAR ligands down-regulate IFN-γ-mediated inflammatory gene expression mainly through attenuation of the JAK-STAT and NFκB pathways. These mechanisms probably act in a ligand- and cell type-specific manner.

1.8.1.5 Transrepression models of PPARs

Studies in the last decade have proposed a number of models that account for the transrepression effect by PPARs. First, co-activator molecules, such as CREB binding protein (CBP) and p300 are shared by NRs and inflammatory transcription factors, such as NFκB, AP-1 and STAT1 (Gerritsen et al., 1997; Liu et al., 2004a; Bouhet et al., 2009). It has thus been suggested that competition for limiting amounts of these proteins represents a mechanism for transrepression by PPARγ and other NRs (Kamei et al., 1996; Li et al., 2000b), and such a mechanism is so-called 'co-activators competition model' (Gerry and Pascual, 2008) (see section 6.1.2 for details).

Additionally, several reports have suggested that PPARs inhibit the expression of inflammatory gene expression by direct interaction with proteins involved in the signal-dependent activation of STAT1, NFκB, AP-1 and C/EBP. Such protein-protein interactions prevent the binding of these transcription factors to their response elements (Pascual and Glass, 2006; Ricote and Glass, 2007; Gerry and Pascual, 2008). For example, Delerive et al. (1999a) showed that in human aortic SMCs, ligand-activated PPARα interferes with AP-1 and NFκB DNA binding acitivity by direct protein interaction with p65 (subunit of NFκB) and c-Jun (subunit of AP-1). The same research group also demonstrated that in human vascular endotheial cells, ligand-activated PPAR-α and -γ alter c-Jun binding to the human endothelin-1 promoter (Delerive et al., 1999).

Recent studies have suggested co-repressor-dependent transrepression models by PPARs. In the case of PPARδ, Lee et al. (2003) proposed a model in which PPARδ

controls the inflammatory status of macrophages based on its association with the transcriptional repressor Bcl-6. In the absence of the ligand, PPARδ sequesters Bcl-6 from inflammatory response genes, such as MCP-1 and IL-1β, resulting in increased gene expression. Once bound to the ligand, PPARδ releases Bcl-6, which in turn exerts anti-inflammatory effects by repressing expression of these genes.

Pascual et al. (2005) suggested that in mouse primary macrophages, PPARγ mediates transrepression of a subset of inflammatory response genes (MIP-1, IP-10, MCP-3 and iNOS) by preventing the signal-specific removal of co-repessor complexes on inflammatory gene promoters downstream of LPS signalling. Ligand activation of PPARγ results in a conformation change allowing the SUMOylation of PPARγ, mediated by the E2 ligase Ubc9 and the E3 ligase, protein inhibitor of activated STAT1 (PIASI). SUMOylated PPARγ binds to the NR corepressor (NCoR)-histone deacetylase-3(HDAC3)-containing co-repressor complex on inflammatory gene promoters, and blocks its degradation by ubiquitylation/19S proteasome pathway, thereby preseving the repressed state. This mechanism has recently been confirmed in mouse peritoneal macrophages by Ghisletti et al. (2007). It will be very interesting to define the extent to which this mechanism is used by other PPAR isoforms and other stimulators. Interestingly, Ghisletti et al. (2007) demonstrated that LXR also uses a SUMOylation and NCoR-dependent pathway to transrepress LPS-mediated inflammatory target gene expression, although the proteins involved in the modification differ. These results suggest that this mechainism may be a general strategy for transrepression by PPARs and LXRs (Ricote and Glass, 2007).

Table 1.7 Transrepression effects of PPARs on IFN-γ-mediated expression of inflammatory response genes

Cell Types	Agonist(s)	PPAR	Genes or proteins analysed	Stimulus	PPAR-dependent or independent	Potential prospective mechanism(s)	References
Mouse RAW264.7 macrophages	15d-PGJ2	γ	iNOS	IFN-γ	independent	inhibition of tyrosine phosphorylation of JAK2, STAT1, DNA binding and transcriptional activity of STAT1	(Chen et al., 2003)
Embryonic stem cell-derived macrophages	15d-PGJ2, BRL49653 ciglitazone troglitazone	γ	iNOS, COX2	IFN-γ	independent		(Chawla et al., 2001)
Peritoneal macrophages	rosiglitazone	γ	iNOS, IP-10, Mig	IFN-γ	partially dependent		(Welch et al., 2003)
Human saphenous vein endothelial cells	15d-PGJ2 troglitazone, BRL49653	γ	IP-10, Mig, I-TAC	IFN-γ	/	inhibition of NFκB DNA binding activity	(Marx et al., 2000)
Mouse mesangial cells	15d-PGJ2	γ	IP-10, Mig, I-TAC	IFN-γ	independent	inhibition of tyrosine phosphorylation of JAK1,2 and STAT1, and nuclear translocation and DNA binding of STAT1	(Panzer et al., 2008)
Mouse astrocytes	15d-PGJ2 rosiglitazone	γ	MCP-1, IP-10, TNF-α, IL-1β, IRF-1	IFN-γ	independent	induction of SOCS-1 and SOCS-3	(Park et al., 2003)

Rat insulinoma RINm5F	15d-PGJ2	γ		IFN-γ	independent	inhibition of STAT1 phosphorylation	(Weber et al., 2004)
Rat insulinoma RINm5F and islets	15d-PGJ2	γ		IFN-γ	/	inhibition of IFN-γ-induced STAT1 phosphorylation, nuclear localisation and DNA binding	(Weber et al., 2003)
Mouse bone-marrow-derived macrophages and primary peritoneal macrophages	15d-PGJ2 troglitazone, rosiglitazone, GW9020	γ	iNOS	IFN-γ, LPS	dependent	inhibition of transcriptional activity of AP-1, STAT1 and NFκB	(Ricote et al., 1998)
Mouse macrophages	15d-PGJ2	γ	NOS2, COX2	IFN-γ+LPS	independent	inhibition of activity of IκB kinase resulting in impaird phosphorylation and degradation of IκBα	(Castrillo et al., 2000)
RAW264.7 mouse macrophages	15d-PGJ2	γ	iNOS, IL-1, COX2	IFN-γ+LPS	/		(Maggi et al., 2000)
Mouse mesangial cell C57BL6 and RAW 264.7 macrophages	GW347845x GW7845 pioglitazone rosiglitazone	γ	iNOS	IFN-γ+LPS	independent		(Crosby et al., 2005)
Mouse astrocytes	15d-PGJ2	γ	TNF-α, IL-1β, IL-6, iNOS	IFN-γ+LPS	independent	inhibition of IκB kinase activity leading to the inhibition of degradation of IκB and nuclear translocation of NFκB (p65);inhibition of PI3K-Akt pathway and recruitment of p300 by NFκB (p65)	(Giri et al., 2004)

Thyrocyte, retrobulbar fibroblasts, retrobulbar preadipocytes	rosiglitazone	γ	IP-10	IFN-γ +TNF-α	/		(Antonelli et al., 2006)
Peritoneal macrophages	GW501516	δ	MCP-1,3,5	IFN-γ, LPS	dependent		(Barish et al., 2008)

Abbreviations: IFN, interferon; TNF, tumour necrosis factor; LPS, lipopolysaccharide; IL, interleukin; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; MCP, monocyte chemotactic protein; NFκB, nuclear factor κB; MIP, macrophage inflammatory protein; COX, cyclooxygenase; IP, interferon-gamma inducible protein; Mig, monokine induced by IFN-gamma; I-TAC, interferon-inducible T-cell alpha chemoattractant; STAT, signal transducer and activator of transcription; IκB, Inhibitor of κB

1.8.2 LXRs

1.8.2.1 Biology of LXRs

Two isoforms of LXR exist (LXRα and β), and they function as cholesterol sensor that regulates both cellular and systemic cholesterol homeostasis (Bensinger and Tontonoz, 2008). The natural ligands for LXRs are sterol metabolites, such as 22(R)-, 20(S)-, 24(S)-hydroxycholesterol and 24(S), 25-epoxycholesterol (Janowski et al., 1996; Janowski et al., 1999). In addition to the endogenous ligands, a number of synthetic pharmacological LXR agonists (i.e. TO901317, GW3965 and acetyl podocarpic) have been reported, which are structurally unrelated to oxysterols (Collins et al., 2002; Sparrow et al., 2002). LXR-α and -β have considerable sequence homology (77% identity in DNA sequence), and seem to respond to the same endogenous ligands, and have almost identical target genes (Bensinger and Tontonoz, 2008; Rigamonti et al., 2008). The mechanisms that regulate LXR gene expression are currently poorly understood. It is, however, interesting to note that human LXRα can autoregulate itself whereas LXRβ cannot. The autoregulation occurs via LXR response elements (LXREs) present in the human LXRα promoter (Laffitte et al., 2001; Whitney et al., 2001). Interestingly, LXRα auto-regulation appears to be limited to human cell types such as macrophages and hepatoma cells and does not occur in murine cells such as primary murine macrophages (Rigamonti et al., 2008). Indeed, important differences have been reported to exist between human and mouse in term of gene regulation by LXRs (Rigamonti et al., 2008 and references therein). As an example, in human macrophages, LXR activation potentiates LPS responses, while no such effect is observed in murine macrophages (Fontaine et al., 2007). Such species-specific action of LXRs indicates that development of a humanised model, such as a murine model that expresses human forms of NRs, represents an attractive strategy for studying LXR biology.

1.8.2.2 LXRs in atherosclerosis

LXRs have the unique ability to promote cholesterol efflux from lipid-laden macrophages, reverse-cholesterol-transport (RCT) to the liver and cholesterol excretion via the bile system [see (Repa et al., 2000; Tobin and Freedman, 2006; Rigamonti et al., 2008) for reviews]. Recently, LXRs have emerged as an important regulator of inflammatory gene expression and immunity. An increasing number of studies clearly demonstrate the potent anti-inflammatory effect of ligand-activated

LXRs in macrophages, the key cells central to lipid homeostasis and inflammatory responses within atherosclerotic lesion formation [(Rigamonti et al., 2008) and references therein]. Indeed, a number of studies on mouse models of atherosclerosis have indicated that LXR activation prevents atherosclerotic development *in vivo*. Joseph et al. (2002) demonstrated that LXR ligands prevent diet-induced atherosclerosis in apoE-/- and LDLR-/- mice. Such athero-protective effect of LXR in LDLR-null mice has been confirmed by Terasaka et al. (2003). Additional investigations have focused on macrophages as the key cellular target for the efficacy of LXR ligands to prevent atherosclerosis. Transplantation of bone marrow from LXR-/- mice into either apoE-/- or LDLR-/- mice increased atherosclerosis, thereby suggesting a protective role of macrophage LXRs in atherosclerotic development (Tangirala et al., 2002). Consistent with this, Levin et al. (2005) further demonstrated that macrophage LXR expression is required for the athero-protective action of LXR agonists using the bone marrow transplantation approach.

Despite the overwhelming evidence supporting the potential benefits of LXR agonists for the treatment of atherosclerosis, the development of an effective and safe LXR agonist-related therapeutic agent is currently facing a major challenge from its role in hepatic lipogenesis. The lipogenic activity of LXR results because of the induced expression of the major regulator of hepatic lipogenesis, SREBP-1c (Repa and Mangelsdorf, 2002) and thereby its downstream targets such as fatty acid synthase (FAS), steroyl CoA desaturase I (SCD-1) and acyl CoA carboxylase (ACC) (Tontonoz and Mangelsdorf, 2003). Thus, pharmacological activation of LXR causes high plasma triglyceride level (Grefhorst et al., 2002), which is clearly a major limitation of using LXR agonists in the treatment of atherosclerosis.

It has become increasingly clear that in addition to its role in regulating cholesterol homeostasis, the athero-protective effect of LXR is also largely dependent on its anti-inflammatory action [see (Bensinger and Tontonoz, 2008; Nomiyama and Bruemmer, 2008) for reviews]. Therefore, understanding the roles of LXRs in inflammatory and immune responses could greatly facilitate our search for the next generation of LXR ligands, which would preferentially exert their protective roles in RCT and inflammation, but exibiting minimal effect on harmful lipogenesis genes.

1.8.2.3 LXRs in inflammation and transrepression

An ever increasing body of evidence suggests that LXRs are an important regulator of inflammatory responses, especially in monocytes/macrophages. Ligand-dependent LXR activation transrepresses the expression of a large range of inflammatory response genes, induced by pro-inflammatory mediators such as LPS, IFN-γ, TNF-α and IL-1β. These genes include chemokines (i.e. MCP-1 and -3, MIP-1β and IP-10), pro-inflammatory cytokines (i.e. IFN-γ, TNF-α, IL-6 and -8), genes important for inflammation response (i.e. COX2 and iNOS), adhesion molecules (VCAM-1) and the genes involved in atheroscleotic plaque rupture and thrombosis (MMP9 and tissue factor) (Castrillo et al., 2003; Joseph et al., 2003; Ogawa et al., 2005; Terasaka et al., 2005; Piraino et al., 2006; Walcher et al., 2006; Birrell et al., 2007; Ghisletti et al., 2007; Myhre et al., 2008) (see Table 1.8 for a full list of publications).

The mechanism underlying the transrepression of inflammatory genes by LXRs is poorly understood. LXRE has not been identified in the proximal promoter of repressed genes at present, thereby suggesting a mechanism distinct from transactivation that is involved in transrepression (Rigamonti et al., 2008). Several lines of evidence indicates that the repression of the NFκB pathway is involved, but maybe via mechanisms independent of inhibition of NFκB nuclear translocation, DNA binding activity or degradation of IκB (Castrillo et al., 2003; Joseph et al., 2003; Terasaka et al., 2005; Piraino et al., 2006; Birrell et al., 2007) (Table 1.8). Although several studies suggested that LXR ligands do not affect the DNA binding and transcriptional activity of AP-1 in mouse peritoneal and human lung tissue macrophages (Castrillo et al., 2003; Joseph et al., 2003; Terasaka et al., 2005; Birrell et al., 2007), Ogawa et al. (2005) showed that in human monocytic THP-1 cell line and RAW264.7 mouse macrophage cell line, LXR ligands inhibit pro-inflammatory cytokine-induced expression of osteopontin by repressing the binding of c-Jun and c-Fos to their AP-1 site in target gene promoters, as well as reducing the expression of c-Jun and c-Fos. Osteopontin is a pro-inflammatory cytokine and adhesion molecule implicated in the chemoattraction of monocytes (Denhardt et al., 2001).

As discussed earlier, SUMOylated PPARγ prevents the LPS-dependent clearance of co-repressors and thus maintains the repressed state of the iNOS promoter (Pascual et al., 2005). A similar but not identical mechanism has also been elucidated for the

transrepression function of LXR. Ghisletti et al. (2007) showed that ligand binding to LXRs results in its SUMOylation by SUMO-2 or -3 and E3 ligase HDAC4 (rather than on SUMO1 and PIAS1 as in the case for PPARγ) for the prevention of clearance of the co-repressor complexes in promoters of inflammatory genes, such as chemokines, iNOS and VCAM-1.

Table 1.8 Transrepression effects of LXRs in inflammatory stimuli-mediated expression of inflammatory response genes

Cell types	Ligands	Genes or proteins analysed	Pro-inflammatory stimuli	Potential prospective mechanisms	References
Human monocytes; human THP-1 cells; mouse RAW264.7 macrophages	GW3965 TO901317	osteopontin	IFN-γ, TNF-α, IL-1β LPS	inhibition of expression of c-Fos and c-Jun; inhibition of binding of c-Fos and c-Jun to AP-1 site of osteopotin promoter	(Ogawa et al., 2005)
J774.2 macrophages	TO901317, 22(R) hydroxy-Cholesterol	iNOS, TNF-α	LPS	inhibition of NFκB activity	(Piraino et al., 2006)
Mouse peritoneal macrophages	GW3965, 22(R)-hydroxy-cholesterol	iNOS, IL-6, MIP-1β, MCP-1 and-3, COX-2, IP-10, IL-1β, SOCS-1 and -3, MMP-9	LPS	inhibition of transcriptional activity of NFκB without affecting that of AP-1	(Joseph et al., 2003)
Human monocytes	GW3965	IL-1β, IL-6, IL-8, TNF-α, MIP-1α, MIP-1β, MCP-1	LPS		(Myhre et al., 2008)
Mouse peritoneal macrophages	GW3965 TO901317	TF	LPS	inhibition of NFκB signalling pathway, without affecting AP-1 transcriptional activity	(Terasaka et al., 2005)

Human lung tissue macrophages; human THP-1 cells	GW3965 TO901317	IL-6, IL-8, MIP-1α, TNF-α	LPS	independent of inhibition of NFκB or AP-1 DNA binding	(Birrell et al., 2007)
Mouse peritoneal macrophages	GW3965	iNOS, MIP-1β, MCP-1, VCAM-1,IL-15	LPS	SUMO-LXR inhibit corepressor NCoR clearance required for gene transcription	(Ghisletti et al., 2007)
Mouse peritoneal macrophages	GW3965 TO901317	MMP9	LPS IL-1β TNF-α	antagonism of NFκB pathway without affecting NFκB nuclear translocation, IkB degradation or DNA binding activity of NFκB or AP-1	(Castrillo et al., 2003)
Human CD4-positive T cells	TO901317	IFN-γ, TNF-α, IL-2	PMA		(Walcher et al., 2006)

Abbreviations: IFN, interferon; LPS, lipopolysaccharide; IL, interleukin; TNF, tumour necrosis factor; PMA, phorbol 12-myristate 13-acetate; MMP, matrix metalloproteinases; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; iNOS, inducible nitric oxide synthase; AP-1, activator protein-1; VCAM, vascular cell adhesion molecule; SUMO, small ubiquitin-related modifier; NcoR, nuclear receptor corepressor; NFκB, Nuclear factor κB; TF, tissue factor

1.9 3-hydroxy-3-methylglutaryl coenzyme A (HMG-coA) reductase inhibitors (Statins) target inflammation

3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors, so-called statins, can largely lower plasma LDL cholesterol levels and are regarded as the most effective cholesterol lowering drugs available. To date, there are many types of statin available including atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. A large number of clinical trials have demonstrated the therapeutic beneficial effects of statins in the prevention of cardiovascular disease, such as atherosclerosis. Although such clinically beneficial effect of statins was initially thought to associate with its cholesterol-lowering effect, a growing body of animal and clinical studies (Davignon and Leiter, 2005; Athyros et al., 2009) have recently provided compelling evidence that statins could exert their athero-protective via actions beyond their effects on serum cholesterol levels [see(Jain and Ridker, 2005; James, 2005; Jasinska et al., 2007; Paumelle and Staels, 2008) for reviews)]. For instance, two groups have independently shown that simvastatin treatment can preserve coronary endothelial function and subsequently moderate atherosclerotic progression independent of lipid lowering actions (Wilson et al., 2001; Bonetti et al., 2002). Such cholesterol-independent effects, so-called 'pleiotropic effects' involves restoration of endothelial function, decrease in oxidative stress, inhibition of vascular inflammation and increase in stability of atherosclerotic plaques [see (Takemoto and Liao, 2001; Lahera et al., 2007; Paumelle and Staels, 2007) for reviews].

1.9.1 An overview of the effects of statins on endothelial dysfunction and inflammation

With regard to the benefits of statin therapy on endothelial function, the pleiotropic actions of statins seem to be directly associated with induced synthesis and activity of eNOS—an enzyme involved in the generation of NO within the vascular wall (Walter et al., 2004; Sugawara et al., 2008; Wang et al., 2008) (Table 1.9). NO confers many beneficial effects such as vascular relaxation and inhibition of leukocyte-endothelial interactions (Jain and Ridker, 2005). In addition, it has been reported that statins inhibit the recruitment of immune cells to the vascular wall through inhibition of pro-inflammatory stimuli-induced expression of adhesion molecules (e.g. VCAM-1 and ICAM-1) (Chung et al., 2002; Takahashi et al., 2005; Montecucco et al., 2009)

and chemokines (e.g. MCP-1 and MIP-1α and -β) (Han et al., 2005; Bruegel et al., 2006; Veillard et al., 2006; Yano et al., 2007; Montecucco et al., 2009) (Table 1.9). The reduction in the expression of adhesion molecules and chemokines, and consequently the cellularity within atherosclerotic lesion have been observed in animal models and human tissues (Aikawa et al., 2001; Crisby et al., 2001; Fukumoto et al., 2001; Kim et al., 2007a; Monetti et al., 2007; Rallidis et al., 2008) .

Statins also show immunomodulatory effects [see (Tristano and Fuller, 2006; Kuipers and van den Elsen, 2007) for reviews]. The drugs were identified to directly reduce the IFN-γ-induced major histocompatibility complex class II (MHC-II) and thus repress MHC-II-mediated T-cell activation. Such inhibition was believed to be attributable, at least in part, to statins-mediated blockage of expression of major histocompatibility class II transactivator (CIITA) (Kwak et al., 2001; Sadeghi et al., 2001; Mach, 2002a; Buttice et al., 2006; Zeinstra et al., 2006; Lee et al., 2008). Additionally, statins suppress the expression of CD40, which plays critical roles in immunological responses, including antigen presentation to T cells, and hence are associated with the adaptive immune response and atherothrombotic complication (Youssef et al., 2002; Mulhaupt et al., 2003; Takahashi et al., 2005; Lee et al., 2007b). Several studies have demonstrated that statins can suppress T cell activation and inflammatory responses by inhibiting the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-2, IL-12 and IL-6 (Youssef et al., 2002; Takahashi et al., 2005). By contrast, treatment of statins has been shown to increase the production of Th2 cytokines, such as IL-4, -5 and -10 (Youssef et al., 2002; Aktas et al., 2003). These results suggest that statins may suppress inflammatory responses at least in part, by promoting a bias from a Th1 response to that of Th2 (see Table 1.9 for a full list of publications).

In addition to its anti-inflammatory and immunomodulatory effects, statins may also benefit atherosclerosis by increasing plaque stability through different mechanisms, such as decreasing macrophage accumulation in atherosclerotic lesions as discussed above, and inhibiting the secretion of MMPs that degrade plaque matrix and, thereby, weaken the fibrous cap (Luan et al., 2003).

Table 1.9 Effect of statins on inflammatory gene expression in vascular cells and atherosclerosis development

Statins	Pathological process	Statin effect	Target genes	Target cells	References
paravastatin, fluvastatin, simvastatin, atorvastatin, lovastatin	EC dysfunction	↓adhesion	↓VCAM-1, ↓ICAM-1	peripheral blood mononuclear cell, EC, SMC	(Chung et al., 2002); (Takahashi et al., 2005); (Montecucco et al., 2009)
pitavasatin, simvastatin	EC dysfunction	↑vasoreactivity	↑eNOS	EC	(Walter et al., 2004); (Sugawara et al., 2008); (Wang et al., 2008)
pitavasatin, simvastatin, atorvastatin	inflammatory cell infiltration	↓macrophages/ T cells recruitment	↓MCP-1, ↓MIP1-α and -β, ↓CCR1,2,4,5	monocytes/macrophages/ T lymphocytes	(Yano et al., 2007); (Montecucco et al., 2009); (Veillard et al., 2006); (Han et al., 2005); (Bruegel et al., 2006)
pitavasatin, simvastatin, atorvastatin, fluvastatin, lovastatin	inflammatory responses	↓pro-inflammatory cytokines, ↓chemokines, ↑anti-inflammatory cytokines	↑CD40, IL-12, ↑IL-1β, IL-6, ↑IFN-γ, TNF-α, ↓IL-4,5,10	monocytes/macrophages/ T lymphocytes, SMC, EC	(Wagner et al., 2002); (Paumelle et al., 2006); (Takahashi et al., 2005); (Yano et al., 2007); (Townsend et al., 2004); (Lee et al., 2007b)

simvastatin, lovastatin, atorvastatin, paravastatin	immune response	↓immunemodulatory molecules	↓MHCII, CIITA	EC, macrophages, SMC	(Kwak et al., 2001); (Sadeghi et al., 2001); (Lee et al., 2008); (Mach, 2002a); (Zeinstra et al., 2006); (Buttice et al., 2006)

Abbreviations: EC, endothelial cells; SMC, smooth muscle cells; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; CCR, CC chemokine receptor; IL, interleukin; IFN, interferon; TNF, tumour necrotic factor; NOS, nitric-oxide synthase
↓, decreased expression; ↑, increased expression

1.9.2 Pleiotropic effects of statins mediated via PPARs

Despite compelling evidence for the pleiotropic effects of statins, the molecular mechanisms underlying their actions remain less understood. Some recent studies have suggested that statins may exert their anti-inflammatory effects via PPARs. Paumelle et al. (2006) demonstrated that in mouse macrophages, statins inhibit the LPS-induced expression of iNOS and IL-6 by enhancing the PPARα transrepression activity on the NFκB pathway. More recently, Yano et al. (2007) showed that statins may exert anti-inflammatory properties via PPARs by generating the ligand, 15d-PGJ_2. In this model, statins induce PPAR-α and -γ activity by increasing the expression of COX2, which converts arachidonic acid into varioius bioactive lipids, such as 15d-PGJ2. These authors additionally showed that statins induce the expression of COX2 by activation of ERK and p38 MAPK pathways. Such MAPK-dependent inhibition of inflammatory gene expression by statins has also been shown by other research groups (Chung et al., 2002; Menschikowski et al., 2005; Montecucco et al., 2009). As discussed earlier, ligand-dependent PPARs suppress the expression of inflammatory genes, at least in part, by antagonising the activity of NFκB and STAT1 (section 1.8.1). Thus the potential statins—MAPK—COX2—15d-PGJ2—PPAR signalling pathway probably provides one of the explanations for the repressive effects of statins on the transcriptional activity of NFκB and STAT1 (Chung et al., 2002; Townsend et al., 2004; Kim et al., 2007b) (Figure 1.5). In addition, the PPARα transrepression properties have been shown to be negatively modulated by PKC signalling in hepatocytes (Blanquart et al., 2004) and the anti-inflammatory effect of simvastatin was suggested to occur via PPARα by a mechanism involving inhibition of the PKC pathway (Paumelle et al., 2006).

Several studies have also suggested that statins might further enhance the mechanisms detailed above by inducing PPAR mRNA expression (Inoue et al., 2002; Roglans et al., 2002; Landrier et al., 2004). Interestingly, a synergistic action of statins and PPARα has been reported on a number of target genes in both macrophages and hepatocytes (Inoue et al., 2002; Landrier et al., 2004; Paumelle et al., 2006). Together, these data support the existence of cross-talk between statins and PPAR signalling pathways (Figure 1.5). The results provide a molecular basis and scientific rationale for the therapeutic association of these drug classes.

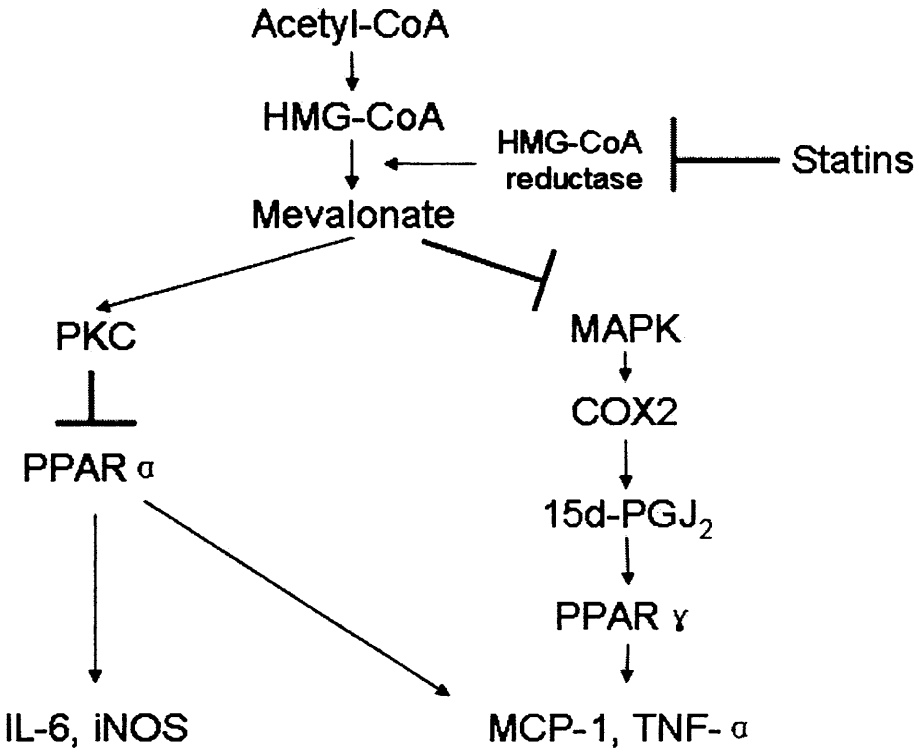


Figure 1.5 Cross-talk of statins and PPARs: anti-inflammatory roles of statins [adapted from (Paumelle and Staels, 2008)]

Statins are competitive inhibitors of the HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. Statins exert their pleiotropic effects via transrepression properties of PPARs, potentially via two mechanisms. Statins induce the expression of COX2 through MAPK pathways. The induced COX2 in turn promotes the generation of PPAR ligands (i.e. 15d-PGJ2), which then activates PPARγ. Alternatively, statins also mediate the inhibition of the PKC pathway, which results in the enhancement of the PPARα transrepession activity. The activated PPARs transrepress the inflammatory response genes (e.g. MCP-1 and iNOS).

Abbreviations: iNOS, inducible nitric oxide synthase; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptors; IL, interleukin; MAPK, mitogen-activated protein kinase; COX, cyclooxygenase; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor

1.10 Aims of the study

Atherosclerosis is recognised as a chronic inflammatory disorder, representing the major cause of mortality in the Western society. Cytokines play crucial roles in the regulation of inflammatory responses during atherosclerosis development, largely through the modulation of gene expression associated with this disease. The study of signalling mechanisms involved in the cytokine-mediated regulation of gene expression implicated in the progression of this disease is vital for the identification of new targets for therapeutic intervention. IFN-γ, an important pro-inflammatory cytokine, acts as a key mediator in atherosclerotic lesion development. The classical JAK-STAT pathway plays a pivotal role in IFN-γ signalling, and of importance, STAT1 Ser727 phosphorylation is known to be essential for its full transcriptional activity. In particular, Ser727 phosphorylation of STAT1 has recently been shown to be important for the IFN-γ-mediated regulation of expression of several key genes implicated in atherosclerosis (Harvey et al., 2007). However, the molecular mechanisms underlying such phosphorylation are yet to be fully elucidated. Thus, the mechanisms underlying STAT1 transcriptional activity, particularly via STAT1 Ser727 phosphorylation stimulated by IFN-γ, is an important area for scientific research.

Recently, a growing body of evidence suggests that ligand-activated NRs (i.e. PPARs and LXRs) and cholesterol-lowering drugs, statins exert atheroprotecctive effects via their crucial anti-inflammtory properties. Despite their well established significance in inflammatory and immune responses, the molecular mechanisms involed in their atheroprotective effects remain largely unknown, particularly in relation to IFN-γ responses, due to limited research on this aspect.

The initial aims of the work presented in this thesis were therefore to:

- Identify the key kinases responsible for the IFN-γ-induced STAT1 phosphorylation, particularly at serine 727 (Chapter 4)
- Investigate the effect of the potential therapeutic agents (i.e. agonists for PPARs and LXRs, along with statins) on the IFN-γ-mediated regulation of gene expression, STAT1 phosphorylation and cellular responses (Chapter 5).

This was to be achieved through the use of RT-PCR and Western blotting, to analyse changes in mRNA and protein levels respectively, combined with the use of specific pharmacological inhibitors, agonists for PPARs and LXRs, and statins. Transient transfection assays and monocyte-endothelium adhesion assays were employed to confirm further the effect of these drugs along with statins on IFN-γ responses.

Initial investigations led to the identification of key roles for protein kinases ERK and JNK in the IFN-γ-mediated STAT1 serine phosphorylation and repressive effects of potential therapeutic agents on IFN-γ-mediated responses. The aims were then extended to include:

- Detailed analysis on the roles of ERK and JNK in the IFN-γ-mediated regulation of gene expression and cellular responses associated with atheroslerosis (Chapter 4)

- Investigate the molecular mechainisms underlying the repressive effects of these therapeutic agents on IFN-γ signalling (Chapter 6)

The roles of ERK and JNK in the IFN-γ signalling pathway were analysed with the use of pharmacological inhibitors and dominant negative mutants. This was achieved by monitoring changes in gene expression, promoter activation, kinase activity and monocyte-endothelium adhesion in response to IFN-γ following treatment of the cells with inhibitors or dominant negative mutants. Moreover, the molecular mechanisms by which the therapeutic agents repress IFN-γ signalling were investigated with the continued use of agonists for PPARs and LXRs, along with statins, by analysing changes in the IFN-γ-induced nuclear accumulation of activated STAT1, STAT1 DNA binding activity and transactivaiton by this transcription factor.

Overall, it was hoped that these studies will significantly contribute to the understanding of the signal transduction pathways activated by IFN-γ and the molecular mechanisms underlying the anti-inflammatory effects of agonists for PPARs and LXRs, along with statins, in relation to IFN-γ responses. Such studies will hopefully contribute to the eventual development of novel therapies to combat atherosclerosis and CAD.

CHAPTER TWO:

MATERIALS AND METHODS

Chapter 2: Materials and methods

2.1 Materials

The materials used throughout the course of this study and the suppliers from which they were purchased are listed in Table 2.1.

Table 2.1 A list of materials and chemicals used in this study with their suppliers.

Materials	Supplies
1-Butanol	Acros, New Jersey, USA
ECL Western Blotting Detection Reagent Rainbow Protein Size Markers Random Hexamers (PdN6)	GE Healthcare, Buckinghamshire, UK
Acrylamide: Bisacrylamide (37.5:1)	Anachem, Luton, UK
10X NH_4 Reaction Buffer Agarose PCR grade Magnesium Chloride Taq Polymerase	Bioline, London, UK
15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2	BIOMOL International, Exeter, UK,
Apigenin Bisindolylmaleimide KN-93 LY294002 PD98059 SB203580 SP600125 Simvastatin (carboxylate form) TO901317	MERCK (Calbiochem), Nottingham,UK
Rosiglitazone (BRL49653) GW7647	Cayman Chemical, Michigan, USA
Falcon 15ml and 50ml Polypropylene Tubes	Corning Costar, Schiphol-Rijk, Netherlands
LB Agar Capsules LB Medium Capsules	DIFCO Laboratories, Surrey, UK
Cell Lines (THP1, Hep3B)	European Collection of Animal Cell Culture (ECACC), Salisbury, UK

EDTA Ethanol Hydrochloric Acid Industrial Methylated Spirits Isopropanol Methanol Sodium Chloride Sodium Hydroxide Tris-Base Other General Chemicals	Fisher Scientific, Loughborough, UK
Kodak X-ray Film	Genetic Research Instrumentation, Essex, UK
D-MEM Tissue Culture Medium with GlutaMAX™ Fetal Calf Serum R.P.M.I 1640 Tissue Culture Medium 0.25% Trypsin-EDTA β-Mercaptoethanol	Gibco BRL, Paisley, UK
1ml Cryo-vials 96 Well Plates Falcon 15ml and 50ml Polypropylene Tubes Tissue Culture Flasks	Greiner, Gloucestershire, UK
Cell Scrapers	Helena Biosciences, Sunderland, UK
1Kb DNA Ladder	Invitrogen, Paisley, UK
HUVEC EGM2 bulletkit	Lonza, Birkshire, UK
High Purity Plasmid Maxi Prep System	Marligen Biosciences, High Wycombe, UK
PVDF Membrane	Millipore Ltd., Gloucestershire, UK
Anti-p38 Antibody Anti-p44/42 Antibody Anti-STAT1 Ser705 Antibody Anti-STAT1 Tyr701 Antibody U0126	New England Biolabs, Hertfordshire, UK
Phosphate-Buffered Saline Tablets	Oxoid Ltd, Hampshire, UK
dNTPs	Promega, Southampton, UK
MMLV 10X Buffer	
MMLV Reverse Transcriptase	
RNasin Ribonuclease Inhibitor	
SuperFECT™ RNeasy MicroKit RNeasy MiniKit	Qiagen, West Sussex, UK
Anti-STAT1 Antibody	Santa-Cruz Biotech Inc., California, USA
Tissue Culture Filters (0.2μM)	Schleicher and Schuell, London, UK
PCR Primers	Sigma Genosys, Cambridgeshire, UK

9-*cis*-retinoic acid 10X TBE Buffer 22(R) hydroxycholesterol 22(S) hydroxycholesterol Ampicillin Anti-β-Actin Benzamidine Bovine Serum Albumin Bromophenol Blue Dimethyl Sulfoxide Molecular Biology Grade DMSO Ethidium Bromide Glycerol Leupeptin Lovastatin (active form) Mineral Oil Penicillin/Streptomycin PMA PMSF Sodium Dodecyl Sulphate Sodium Fluoride Sodium Orthovanadate Sodium Pyrophosphate TBE (x10) Tissue Culture Grade DMSO Trypan Blue Solution	Sigma, Poole, UK
Interferon-γ	Totam Biologicals (Tebu Bio), Cambridgeshire, UK

2.2 Preparation of solutions, glass-and plastic-ware

Solutions, glass- and plastic-ware used for RNA/DNA and tissue culture related work were autoclaved at 121°C (975kPa) for 20-30min.

2.3 Cell culture techniques

2.3.1 Cell lines

The cell lines utilised for the work detailed in this study were human monocytic leukemia cell line, THP-1 and human hepatoma cell line, Hep3B. THP-1 cells are undifferentiated monocytes grown in suspension (derived from a human monocytic leukaemia) (Tsuchiya et al., 1980). This cell line provides a valuable model for studying the physiological and biochemical properties displayed by macrophages along with the regulation of gene expression in these cells because following differentiation with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), they mimic native monocyte-derived macrophages in several aspects (Tsuchiya et al., 1982; Auwerx, 1991). Hep3B cells are fully adherent hepatocytes derived from a human carcinoma (Aden et al., 1979).

2.3.2 Maintenance of cell lines in culture

THP-1 cells were grown in R.P.M.I-1640, and Hep3B cells were grown in DMEM. All media were supplemented with 10% (v/v) heat-inactivated (30min, 56°C) fetal calf serum (HI-FCS) in the presence of penicillin (pen, 100U/ml) and streptomycin (strep, 100µg/ml). Penicillin, streptomycin and HI-FCS were filter-sterilised by passing through 0.2µm sterile filters prior to use.

2.3.3 Subculturing of cell lines

2.3.3.1 THP-1 cells

The suspension cells were subcultured when they reached approximately 60% confluence. The cells were transferred into polypropylene tube (Falcon) and

centrifuged at 110g for 5min. Following the aspiration of the medium from the cells, the pellet was re-suspended in fresh R.P.M.I-1640 medium with 10% (v/v) HI-FCS at a ratio of 1:6 (i.e. cells from one tissue culture flask were subcultured into 6 new flasks). The cells were then grown up at 37°C in a humid incubator with an air mixture containing 5% (v/v) CO_2 in air.

2.3.3.2 Hep3B cells

Adherent Hep3B cells were subcultured when they reached approximately 70% confluence. The cells were first washed once with antibiotic-and HI-FCS-free DMEM medium. Cells were then treated with appropriate volume of 0.25% trypsin-EDTA solution (2, 5 and 10 ml for 25, 75, 125mm^2 flasks, respectively) and incubated at 37°C (5% (v/v) CO_2) for approximately 2-5min until the cells were visibly detached from the substratum. Cells were then re-suspended in an appropriate volume of complete medium (10% (v/v) HI-FCS) and centrifuged at 110g for 5min (FCS contains proteins that neutralize trypsin). The cell pellet was then re-suspended in an appropriate volume of complete medium (10% (v/v) HI-FCS) and seeded into tissue culture flasks at a ratio of 1:4-1:6 (i.e. cells from one tissue culture flask were subcultured into 4-6 new flasks).

2.3.4 Counting cells

A haemocytometer (Neubauer Chamber, GMBH & Co., Wertheim, Germany) was used to count the cells. The haemocytometer was covered with a precision ground coverslip and 8µl of cell suspension was placed at the edge of the coverslip. The cells within the large square were then counted (total number of cells/ml=cells in the large counting square x 10^4).

2.3.5 Preservation and storage of cell lines

Only cells of an early passage (normally less than 2) were preserved. Adherent cells were first trypsinised using trypsin-EDTA. Following centrifugation of trypsinized

Hep3B cells and THP-1 suspension cells at 110g for 5min, cell pellets were re-suspended at a density of approximately $5x10^6$ cells/ml in HI-FCS with 10% (v/v) of either filter-sterilised glycerol (for THP-1) or DMSO (for Hep3B). The cell suspension was aliquoted into 1ml cryo-ampoules, which were frozen down slowly at -80°C in an insulated box overnight. The cells were then transferred into a liquid nitrogen container.

2.3.6 Thawing of frozen cell lines

Frozen cells were thawed in a 37°C water-bath and then transferred to a centrifuge tube containing 15ml of appropriate medium supplemented with 10% (v/v) HI-FCS. Following centrifugation at 110g for 5min, the cell pellets were resuspended in 15-20ml of appropriate medium supplemented with 20% (v/v) HI-FCS. The suspension was then transferred into tissue culture flasks and cultured as normal.

2.3.7 Treatment of THP-1 cells with PMA

THP-1 cells were prepared and cultured in tissue culture flasks or multiple-well plates for 4h (sections 2.3.2-3). The suspension monocytes were then differentiated into macrophages by addition of 0.16μM PMA followed by incubation for 24h.

2.3.8 Treatment of cells with inhibitors or therapeutic drugs and IFN-γ

PMA-differentiated THP-1 macrophages were cultured as normal. When Hep3B cells reached approximately 70% confluence, the cells were washed with fresh DMEM 4h before treatment with the effectors. Inhibitors or therapeutic drugs were added to the cell culture medium for appropriate period of time prior to the addition of IFN-γ at 1000U/ml for THP-1 macrophages and 100U/ml for Hep3B cells. Control cells were treated with an appropriate volume of a vehicle, typically DMSO. The cells were incubated with IFN-γ for the appropriate period of time after which RNA or protein extraction was carried out. Table 2.2 lists the pharmacological inhibitors used and their mechanisms of action.

Table 2.2 Pharmacological inhibitors with concentrations used in this study and their mechanisms of actions

Inhibitors	Chemical name	Target	Mechanism of action	Conc. Used (μM)	References
KN-93	2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine	CaMKII	Competitive binding to calmodulin binding sites of the enzyme and preventing the association of calmodulin with CaMK II; also affects calcium channels in a CaMKII-independent manner	5,10,20	(Sumi et al., 1991)
Apigenin	4',5,7-Trihydroxyflavone	CK2	Reversible ATP/GTP-competitive inhibitor of CK2	5,10,20	(Critchfield et al., 1997)
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene	ERK1/2/5	Reversible allosteric inhibitor of inactive forms of MEK1/2/5 and hence prevents the activation of downstream kinases, ie. ERK1/2/5	5,10,20	(Favata et al., 1998)
PD98059	2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one	ERK1/2/5	Reversible allosteric inhibitor of inactive forms of MEK1/2/5 and hence prevents the activation of downstream kinases, ie. ERK1/2/5	5,30,50	(English and Cobb, 2002)
SP600125	1,9-Pyrazoloanthrone	JNK/SAPK	Reversible ATP-competitive inhibitor of JNK1,2,3 isoforms with similar potency	10,15,30	(Bennett et al., 2001)

SB203580	4-(4-fluorophenyl)-2-(4-methylsulfinylpheny)-5-(4-pyridyl)1H-imidazole	p38	Reversible ATP-competitive inhibitor to active form of p38 MAPK; Non-ATP-competitive inhibitor to inactive form of p38 kinase by preventing the binding of the enzyme to ATP and hence inhibiting kinase activation *in vivo*.	5,10,30	(Frantz et al., 1998)
LY294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one	PI3K	Reversible ATP-competitive inhibitor of PI3K	10,20,30	(Vlahos et al., 1994)
Rottlerin	5,7-dihydroxy-2,2-dimethy-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene	PKCδ	Reversible ATP-competitive inhibitor of PKCδ at low concentrations (IC_{50}=3-6μM)	3,6,10	(Gschwendt et al., 1994)

Abbreviation: CamK II, calcium camodulin-dependent protein kinase II; CK2, casein kinase 2; ERK, extracellular-regulated kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; Conc. Concentrations

2.3.9 Delipidation of HI-FCS

In order to prevent the potential interference of responses studied by fatty acids and other lipids that might exist in FCS (activation of LXRs and PPARs), HI-FCS was further delipidated for the studies on NRs by the method of Cham and Knowles (1976). Thus, HI-FCS (10ml) was mixed with 8ml of 1-butanol and 12ml of diisopropyl ether (DIPE) by rotation for 60min at room temperature. The serum phase (aqueous phase at the bottom of the centrifuge tube) was separated from lipids and the organic solvent by centrifugation at 2300g for 10min at room temperature, and then transferred into a fresh Universal tube using a syringe with needle. The residual solvent in serum was removed by blowing nitrogen bubbles through it for 5-6h until the solvent's odour had disappeared. The delipidated FCS was filter-sterilised by passing through a 0.2μm sterile filter and stored at -20°C until required.

2.3.10 Primary culture of Human Umbilical Vein Endothelial Cells (HUVEC)

The primary endothelial cells utilised for leukocyte-endothelium adhesion assay (section 2.8) were HUVEC (pooled, cryopreserved cells obtained from Lonza, (Berkshire, UK). The cells were cultured in EGM medium (Bulletkits) and prepared for experiments according to the instructions detailed by the provider, with minor modifications. Thus, 15ml of EGM medium was pre-warmed in a 75cm^2 flask in a 37°C, 5% CO_2 humidified incubator for at least 30min. Frozen cells were defrosted in a 37°C water bath straight after being taken out from liquid nitrogen and quickly dispensed into this culture vessel. The growth medium was changed everyday after seeding and more volume of medium was added as the cells became more confluent: 1ml per 5cm^2 at ~25% confluency, 1.5ml per 5cm^2 at between 25-45% confluency and 2ml per 5cm^2 for over 45% confluency.

The cells were then prepared for experiments (section 2.8) or sub-cultured when they were 70-80% confluent. For each 25cm^2 of cells to be cultured, they were first

washed once with 10ml pre-warmed HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered saline solution (HEPES-BSS), and subsequently treated with 2ml pre-warmed trypsin-EDTA solution and incubated at 37°C for approximately 10-15sec until the cells were visibly detached from the substratum. Cells were then re-suspended in 4ml of pre-warmed trypsin neutralising solution and centrifuged at 110g for 5min. The cell pellet was then re-suspended in 3ml of EGM and cells counts and viability were determined (section 2.3.12). An appropriate volume of fresh EGM was then added to the suspension to make the seeding density of 2000-3000 cells/cm^2 (1ml of growth medium for every 5cm^2 surface area of the flask).

2.3.11 Human primary monocyte-derived macrophages

Human blood buffy coats were obtained from the National Blood Service Wales. The buffy coat was underlayered with Lymphoprep (Nycomed Pharma) (2:1 (v/v) buffy coats:Lymphoprep), which contains dextran and sodium diatrizoate to cause erythrocyte aggregation, and subjected to centrifugation (800g, 30min) to allow sedimination of erythrocytes. The resulting interface was collected and washed with an equal volume of phosphate buffered saline (PBS)-0.4% (w/v) tri-sodium citrate. Cells were pelleted by centrifugation (800g, 5min), resuspended in 0.2% (w/v) saline (sodium chloride) solution and incubated on ice (30sec). An equal volume of 1.6% (w/v) saline (sodium chloride) solution was added and the cells pelleted immediately by centrifugation (800g, 5min). The pellet was then washed 6-8 times with PBS-0.4% (w/v) tri-sodium citrate to remove contaminating platelets. The remaining cells were plated in R.P.M.I 1640 (5% HI-FCS and 1% (v/v) pen/strep) and incubated overnight at 37°C, 5% (v/v) CO_2 in order to allow the adherence of monocytes. After this time, half of the medium was replaced with fresh complete medium. Culture medium was replaced every 4 days for 7-10 days, over which time the cells differentiated from monocytes to macrophages.

2.3.12 Cell viability assays

2.3.12.1 Trypan blue exclusion assay

The procedure was adapted from that by Lecaroz et al. (2006). Cells were counted, cultured and differentiated as normal in 8cm^2 cell culture dishes (sections 2.3.2-4). The incubation medium was removed from the cells, which were then washed with antibiotic- and HI-FCS-free R.P.M.I-1640 and detached with typsin-EDTA. Trypan blue solution (0.4% (w/v)) was added to the cell solution at a ratio of 1:1 (v/v) and incubated for 1-2min. The number of stained and total cells was counted using a hemocytometer as detailed in section 2.3.4. The calculated percentage of unstained cells represents the percentage of viable cells.

2.3.12.2 ViaLight® plus assay

The assay was performed according to manufacturer's instruction of the kit (Lonza). All the reagents in the kit [i.e. cell lysis reagent, ATP monitoring reagent plus (AMR PLUS), assay buffer)] were gently allowed to reach room temperature. The tissue culture plates were removed from the incubator and left to cool down to room temperature for 5min. Cells were then lysed with 50µl of cell lysis buffer for 10min and 100µl AMR PLUS assay buffer was then added to each well of the plates. After incubation for 2min, living cells was quantified by measuring luminescence using FluoStar Optima (BMG LABTECH, Aylesbury, UK) with 1sec integrate period.

2.3.12.3 MTT-based toxicology assay

The assay was performed according to instructions by the manufacturers (Sigma). Thus, MTT solution (10% (v/v) of the culture medium volume) was added directly to the cells with medium, and then incubated as normal for 2h. Following the incubation period, the plates were removed from the incubator and the resulting formazan crystals were dissolved in MTT solubilisation solution using an amount that was equivalent to the original culture medium volume. The absorbance of the resulting solution was measured spectrophotometrically at a wavelength of 570nm.

2.4 RNA/DNA-related techniques

2.4.1 RNA isolation

Total RNA was isolated from cells using the RNeasy kit (Qiagen). Adherent cells were detached by using a cell scraper, and the mixture was then transferred into a polypropylene tube (Falcon) and centrifuged at 110g for 5min. The pellets were lysed by the addition of RLT buffer (provided in the kit) supplemented with 10µl/ml of β-mercaptoethanol, and the suspension was homogenised by passing several times through a 0.9mm needle. The rest of the steps were carried out according to the manufacturer's instructions.

The concentration and purity of RNA was determined using U-1500 Hitachi spectrophometer by measuring the absorption at 260nm and 280nm (O.D. 260 and O.D. 280, respectively). The quality of RNA (1µg) was also analyzed by electrophoresis on a 1% (w/v) agarose gel (section 2.4.3).

2.4.2 Reverse Transcriptase PCR (RT-PCR)

RT-PCR consisted of two steps:

1. cDNA was synthesised from isolated RNA using reverse transcriptase (section 2.4.2.1)

2. Standard PCR reaction was performed using gene-specific primers to obtain a specific product (section 2.4.2.2)

2.4.2.1 Reverse Transcription

RNA (typically 1µg) was incubated with random hexamers (PdN6, 200pmol) in a volume of 13.5µl (made up with sterile water) at 72°C for 5min and then cooled immediately on ice. Subsequently, the following reagents were added into the reaction mixture:

1µl of dNTP mixture (10mM each of dATP, dGTP, dCTP and dTTP);

1µl of 200U of M-MLV (Molony Murine Leukemia Virus) reverse transcriptase;

0.5µl of 40U/ml recombinant RNase inhibitor;

4µl of 5X reverse transcriptase buffer

The total mixture (20µl) was then incubated at 37°C for 1h and the reaction was terminated by incubation at 94°C for 2min. The resultant cDNA was then diluted 5-fold by adding 80µl nuclease-free water and stored at -20°C.

2.4.2.2 PCR

The primer sets used in the PCR reactions are shown in Table 2.3. PCR reactions contained 1x buffer (Bioline), dNTPs (100µM), Taq polymerase (1U) and forward and reverse primers (1µM). The concentration of magnesium chloride ($MgCl_2$) and cDNA was varied according to initial optimisation experiments, and DMSO was used for some primer sets (Table 2.4). Molecular grade water was added to bring the final volume of the reaction to 50µl.

Table 2.3 Sequences of forward and reverse primers used in PCR and their product sizes in base pairs (bp)

Primer names	Forward primer	Reverse Primer	Product size	References
28S rRNA	5'-TTAGACCGTCGTGAGACAGG-3'	5'-TTCAATAGATCGCAGCGAGG-3'	510	(Gibbs et al., 2002)
β-actin	5'-ATGATATCGCCGCGCTCG-3'	5'-CGCTCGGTGAGGATCTTCA-3'	580	/
CIITA pIII	5'-GCCCTGCTGGGTCCTACCTG-3'	5'-GAACTGGTCGCAGTTGATG-3'	215	(Piskurich et al., 2006)
CIITA pIV	5'-AGGGAGAGGCCACCAGCAG-3'	5'-GAACTGGTCGCAGTTGATG-3'	227	(Zhao et al., 2007)
GAPDH	5'-CCCTTCATTGACCTCAACTACATGG-3'	5'-AGTCTTCTGGGTGGCAGTGATGG-3'	455	(Sabatakos et al., 1998)
ICAM-1	5'-AATGCCCAGACATCTGTGTC-3'	5'-CAGTTCAGTGCGGCACGAGAAAT	521	/
ICER I/II	5'-CTGATGAGGAAACTGAACTTG-3'	5'-TCGGCTCTCCAGACATTTTAC-3'	657/257	(Peri et al., 2001)
IP-10	5'-CCACGTGTTGAGATCATTGC-3'	5'-ACATAGCACCTCAGTAGAGC-3'	383	(Berg et al., 2006)
MCP-1	5'-CTTCTGTGCCTGCTGCTCATAGCA-3'	5'-CTTGGGGTCAGCACAGATCTCCTT-3'	219	/
MIP-1β	5'-GGAAGCTTCCTCGCAACTTTG-3'	5'-GCTCAGGTGACCTTCCCTGAA-3'	200	(Chiba et al., 2004)
SOCS-1	5'-AGACCCCTTCTCACCTCTTG-3'	5'-CTGCACAGCAGAAAAATAAAGC-3'	202	/

Table 2.4 Reaction conditions for the amplification of various PCR products

Genes	dNTPs (10mM) µl	$MgCl_2$ (50mM) µl	DMSO µl	cDNA µl	Initial melting	Annealing	Extension	Melting	Final long extension	No. of cycles
28S rRNA	1	2	5	10	94°C 2min	62°C 30sec	72°C 1min	94°C 30sec	72°C 10min	12
β-actin	1	1.5	2.5	10	95°C 5min	57°C 1min	72°C 2min	93°C 30sec	72°C 10min	16
CIITA pIII	1	1	2.5	10	96°C 5min	60°C 1min	72°C 1min	96°C 1min	72°C 10min	25
CIITA pIV	1	1	-	10	96°C 5min	60°C 1min	72°C 1min	96°C 1min	72°C 10min	31
GAPDH	0.5	2	-	10	96°C 5min	60°C 1min	72°C 2min	93°C 30sec	72°C 10min	18
ICAM-1	0.5	2	-	5	96°C 5min	64°C 30sec	72°C 1.5min	96°C 40sec	72°C 10min	25
ICERI/II	1	1.5	-	5	95°C 2min	56°C 1min	72°C 2mim	95°C 30sec	72°C 5min	27
IP-10	1	1	-	10	95°C 15min	56°C 45sec	72°C 1min	94°C 45sec	72°C 10min	16
MCP-1	0.5	1.5	-	10	92°C 2min	60°C 1min	72°C 2min	95°C 30sec	72°C 10min	17
MIP-1β	1	1	5	10	50°C 2min; 95°C 15min	60°C 30sec	72°C 1min	95°C 15sec	72°C 10min	34
SOCS-1	1	1.5	2.5	5	94°C 5min	58°C 1min	72°C 1min	94°C 1min	72°C 10min	27

Abbreviations: CIITA: MHC class II transactivator; GAPDH: Glyceraldehye-3-phosphate dehydrogenase; ICAM: intercellular adhesion molecule 1; ICER: inducible cAMP early repressor; IP-10: interferon-gamma inducible protein-1; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein; SOCS: suppressor of cytokine signaling

2.4.3 Agarose gel electrophoresis of RNA/DNA

Size fractionation of PCR products and RNA was carried out by agarose gel electrophoresis. Gels were made from agarose dissolved in 1x TBE buffer containing 0.5μg/ml of ethidium bromide. The following stock solutions were used for electrophoresis:

10x TBE;

5x DNA loading dye (1x TBE, 50% (v/v) glycerol and 2.25% (w/v) bromophenol blue);

RNA loading dye stock (1M EDTA, 50% (v/v) glycerol and 2.25% (w/v) bromophenol blue)

2.4.3.1 Resolving RNA on agarose gels

RNA (1μg) was resolved on 1% (w/v) agarose gels to assess its integrity. RNA samples were prepared with 3μl of sterile RNA loading dye and made up to a final volume of 18μl with RNase free water (Qiagen). Electrophoresis was carried out in 1x TBE buffer using a Fisherbrand Horizonal Gel Unit at 100V for approximately 10-15min

2.4.3.2 Resolving PCR products and plasmid DNA on agarose gels

Agarose gels (1.0-2.0% (w/v)) were prepared depending on the size of the PCR products (the larger the size of DNA, the lower the concentration of agarose used). The size of PCR products were determined by comparison with standard DNA molecular markers (see Appendix I). Routinely, DNA samples were prepared with 10μl of 5x DNA loading dye to achieve a final 1x DNA loading dye concentration. Electrophoresis was carried out on a Fisherbrand Horizontal Gel Unit at 100-200V for 30-60min in 1x TBE buffer.

2.4.3.3 Densitometric analysis

Following electrophoresis, RNA/DNA was visualized under UV light using a Syngene Gel Documentation System. The signals were quantified using Quantiscan computer package (Biosoft, Cambridge, UK).

2.4.4 Bacterial strains and vectors

All bacterial culture media (LB-agar and LB-liquid media) were prepared according to the manufacturer's instructions (DIFCO Laboratories). Table 2.5 indicates the genotype of the *Escherichia Coli* (*E. Coli*) strains used in this work. Plasmid vector maps can be found in Appendix II.

Table 2.5 Genotypes of *E.Coli* strains used in this study

Strain	Genotype	Reference
DH5α	supE44ΔlacU169(ϕ80lacZΔM15)hsd17 recA1endA1gyrA96thi-1relA1	(Hanahan, 1983)

2.4.4.1 Transformation of competent cells

Plasmid DNA (1μg) was added to 200μl of competent cells, which were subsequently incubated on ice for 30-40min. After being heat shocked at 42°C for 90sec the cells were immediately placed for 2min on ice. LB medium (800l) (pre-warmed to 37°C) was added to the cells and incubated at 37°C with shaking (300r.p.m.) for 1h. Transformed bacteria (200μl) were spread onto agar plates containing ampicillin (100g/ml). The remaining cells were pelleted by microcentrifugation (3000g, 3min), resuspended in LB medium (200μl) and spread onto separate plates. Colonies of transformed bacteria (ampicillin resistant) were grown overnight at 37°C.

2.4.4.2 Preparation of plasmid DNA (maxi-prep)

LB medium (15ml) containing ampicillin (100g/ml) was inoculated with a single colony of transformed *E.Coli* and incubated at 37° C for 7h with constant shaking (300r.p.m.). Then, 1ml of this bacterial culture was inoculated into fresh LB-medium (500ml) containing ampicillin (100μg/ml) and incubated overnight at 37°C with shaking (300r.p.m.). Plasmid DNA was subsequently prepared using a High Purity Plasmid Purification Maxiprep kit according to the manufacturer's instructions (Marligen Biosciences). The plasmid DNA was diluted in sterile molecular biology grade water following purification. The concentration and purity of the DNA was determined by measuring O.D.260 and O.D.280 using a U-1800 Hitachi spectrophotometer.

2.4.4.3 Restriction endonuclease digestion of recombinant plasmid DNA

DNA (0.5μg) was mixed with the restriction endonuclease and its appropriate buffer (recommended by the enzyme's supplier New England Biolabs) in a total volume of 20μl. The digests were typically carried out using 10U of each enzyme with the exception of KpnI where 20U were used due to its low activity. BSA (1x) was added where required. The reaction mixture was incubated at 37°C for 2h, unless otherwise stated. All reactions were terminated by incubation at 65°C for 10min. The DNA fragments were analysed by agarose gel electrophoresis (section 2.4.3).

2.5 Transfection of cells with DNA

2.5.1 Plasmids used for transfection-based assays

2.5.1.1 MCP[213]Luc

The luciferase reporter construct MCP[213]Luc was donated by Dr. R. M. Ransohoff (Cleveland Clinic Foundation). A region of the human MCP-1 promoter 213bp upstream of the transcriptional start site was inserted into the pGL2-Basic luciferase reporter vector (Promega) (Zhou et al., 1998).

2.5.1.2 3xly6e

The construct 3xly6e was donated by Prof J E Darnell (Rockefeller University, New York). The promoter contains 3 tandem STAT1 consensus sites (GAS) regulating the expression of a luciferase reporter gene in the pZLuc-TK vector.

2.5.1.3 DN JNK/SAPK

The DN JNK/SAPK plasmid, SAPKα-VPA, was generated by changing the phosphorylation site Thr-Pro-Tyr to Val-Pro-Ala (Kawasaki et al., 1996).

2.5.1.4 DN ERK1 (K71R) and ERK2 (K52R)

The ERK1 and ERK2 DN plasmid constructs, K71R and K52R respectively, were a generous gift from Prof. M.H.Cobb (UT Southwestern Medical Center, Dallas, Texas, U.S.A). The constructs contain kinase inactive mutants (lysine to arginine change at residues 71 and 52 for ERK-1 and -2 respectively) in the plasmid vector pCEP4 (Invitrogen) and p3XFLAG-CMV-7.1 (Sigma) for ERK-1 and -2 respectively (Frost et al., 1994).

2.5.1.5 DN Ras and Raf-1

The Ras (Asn-17) and Raf-1 (Ala-375) DN plasmid constructs were a kind gift from I. Komuro of Tokyo University Medical School (Zou et al., 1996).

2.5.1.6 DN MEK1

Plasmids encoding dominant-negative (pCMV-MEK-2A) form of MEK1, in which serine-218 phosphorylation sites were modified to alanine, were kindly provided by Prof. J. Han (Scripps Research Institute, California, US) (Yan and Templeton, 1994).

2.5.1.7 SRC-1 and p300

pSP64-p300 and pSP64-SRC-1 in the pSP64(polyA) vector were constructed by cloning the full-length cDNA encoding human p300 or SRC-1 respectively (Li et al.,

2000c). They were both generously given by D M Livingston (Dana-Farber Cancer Institute, Boston, MA, USA).

2.5.1.8 LXR-α and -β expression plasmids

LXR-α and LXR-β expression plasmids were a generous gift from Prof. Steven A. Kliewer (Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas, U.S.A). The cDNAs encoding the human LXR-α or LXR-β was inserted into the expression vector pSG5 (Lehmann et al., 1997).

2.5.1.9 DN LXR-α plasmid

The DN LXR-α plasmid was a generous gift from Prof. Thomas A. Kocarek (Institute of Environmental Health Sciences, Wayne State University, Detroit, U.S.A.). The construct contained the full length sequence of the mouse LXR-α lacking the C-terminal 10 amino acids and the AF-2 subdomain in the pSG5 expression vector (Kocarek et al., 2002).

2.5.2 Transfection using SuperFECT™

SuperFect Reagent consists of molecules ended with charged amino groups which can interact with negatively charged phosphate groups of DNA. SuperFect Reagent-associated DNA can then bind to the cell membrane and are taken into the cell by endocytosis.

To prepare the cells for transient transfection, Hep3B were split at a ratio of 1:4-1:6 and seeded at equal numbers in 12-well plates (1ml of complete DMEM medium in each well). The cells were incubated until they reached approximately 70% confluence (section 2.3.3.2). The medium was withdrawn and replaced with new fresh complete medium (0.5ml for each well) prior to transfection. The transfection method was carried out essentially as described in the manufacturer's protocol (Qiagen). The DNA/SuperFect complex was prepared by diluting DNA (1-2μg) in

50µl DMEM (without HI-FCS and antibiotics). SuperFect solution (3.5µl/µg DNA) was added and the mixed DNA-SuperFect solution was incubated at room temperature for 10min. The complex was then resuspended in DMEM complete medium (500µl/µg DNA) and added dropwise to the cells. The cells were then incubated for 1h prior to the further treatment with the appropriate effectors.

2.5.3 Preparation of cell extracts for measurement of luciferase activity

The cells were washed once with PBS. Then, 120µl of 1x passive lysis buffer (Promega) was added directly to the cell monolayer and left to incubate for 10min at room temperature. The cells were then scraped into the buffer, transferred into a microcentrifuge tube, vortexed for 45sec and centrifuged at 9,600g for 3min. The supernatant was transferred into a fresh tube and was either stored at -80°C or used immediately for the measurement of luciferase activity.

2.5.4 Measurement of luciferase activity

Extracts from transfected cells (60µl) were mixed with the luciferase substrate (100µl) and the fluorescent intensity (counts) was determined using a Turner Designs 50/50 Luminometer set at a sensitivity value of 70% with a 2sec delay period and 20sec integrate period. The recorded number of counts was relative to the amount of luciferase expressed by the reporter plasmid and hence the activity of the promoter sequence of interest. All measurements were taken in duplicate and the background values using the luciferase substrate (100µl) alone were subtracted from the sample values. Counts were normalised to the concentration of the proteins (µg/ml) in each lysate, which were determined as described in section 2.6.5.

2.6 Protein analysis

2.6.1 Stock solutions

Table 2.6 A list of the stock solutions used for protein analysis

Solution	Composition
Bromophenol blue solution	0.05% (w/v) bromophenol blue in H_20
Laemmli sample buffer	0.125M Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) β-2-mercaptoethanol
Phosphatase-free whole cell extraction buffer	10mM Tris-HCl (pH 7.05), 50mM NaCl, 50mM NaF, 1% (v/v) Triton X-100, 30mM sodium pyrophosphate, 5μM $ZnCl_2$, 100μM sodium orthovanadate, 1mM DTT, 2.8μg/ml aprotinin, 2.5μg/ml each of leupeptin and pepstatin, 0.5mM benzamidine, 0.5mM PMSF
SDS-PAGE lower gel buffer	1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS, pH 6.8
SDS-PAGE upper gel buffer	1M Tris-HCl (pH 6.8), 10% (w/v) SDS pH 8.8
SDS-PAGE running buffer	25mM Tris, 250mM glycine, 0.1% (w/v) SDS pH 8.3
SDS-PAGE gel loading buffer (reducing)	50mM Tris-HCl (pH 6.8), 100mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol
Western blot transfer buffer	25mM Tris, 192mM glycine, 20% (v/v) methanol
Whole cell extraction buffer	10mM HEPES (pH 7.9), 400mM NaCl, 0.5mM DTT, 5% (v/v) glycerol, 0.5mM PMSF, 10μg/ml aprotinin, 0.5M benzamidine, 0.1mM EDTA
10x Tris-buffered saline (TBS)	10mM Tris-HCl, 20mM NaCl, pH 7.4

2.6.2 Preparation of protein extracts using the Laemmli sample buffer

The cells were washed with PBS and freshly prepared Laemmli sample buffer (70μl/well for 6-well plate; 150μl for 40-50ml flask; 400μl for 250-300ml flask) was added directly to the cells, which were then scraped directly into this buffer. The extracts were then collected by microcentrifugation (17,000g, 3min). At this point, the extracts were either frozen (-80°C) for use on a later date or subjected to SDS-

polyacrylamide gel electrophoresis (SDS-PAGE) (section 2.6.7) and western blotting (sections 2.6.8).

2.6.3 Preparation of phosphatase-free whole cell protein extracts

Cells were scraped into the medium, transferred to a polypropylene tube (Falcon) and centrifuged at 1000g for 5min. The cell pellet was washed twice with ice-cold PBS containing 10mM NaF and 100μM sodium orthovanadate (1ml), and microcentrifuged (17,000g, 1min) between washes. The supernatant was discarded and the cell pellet was resuspended in freshly prepared phosphatase-free whole cell extraction buffer (Table 2.6) (100μl for 25cm^2 flask; 200μl for 75cm^2 flask) by vigorous pipetting and vortexing (45sec). Following centrifugation (10,000g for 10min at 4°C), the supernatant, containing the total cellular protein, was removed and stored at -80°C. The concentration of total cellular protein was determined using the Micro BCA protein assay reagents (Pierce) (section 2.6.5).

2.6.4 Preparation of nuclear/cytoplasmic protein extracts

Nuclear and cytoplasmic extracts were prepared using the Nuclear Extract Kit from Active Motif (Rixensart, Belgium) according to manufacturer's protocol. The volume of reagents stated below is applicable to protein extracted from approximately 8 million cells. After washing once, the cells were scrapped into ice-cold PBS/phosphatase inhibitor and subjected to centrifugation (4°C, 800g, 5min). The pellet was resuspended into 500μl of 1x hypotonic buffer (provided in the kit) and incubated on ice for 15min. Then, 25μl of detergent (provided in kit) was added to the mixture, which was then subjected to vortexing for 10sec followed by micrcentrifugation for 30sec (13,000g, 4°C). The supernatant (cytoplasmic fraction) was stored at -80°C until required. The nuclear pellet was resuspended in 50μl of complete lysis buffer [supplemented with 10mM DTT and protease inhibitor cocktail (provided in kit)] by vortexing for 10sec. The suspension was incubated on ice for 30min and then vortexed for 30sec. Following microcentrifugation (10min at

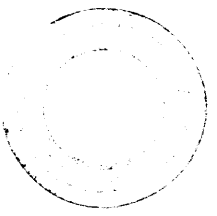
13,000g, 4°C), the supernatant (nuclear fracton) was aliquoted, in order to avoid freeze/thaw cycles, and stored at -80°C. The concentration of protein fractions was determined using the Micro BCA protein assay reagents (Pierce) (section 2.6.5).

2.6.5 Determination of protein concentration

The concentration of proteins in extracts was determined using the Micro BCA Protein Assay Reagent Kit (Pierce) according to the manufacturer's instructions. A standard curve of absorbance versus concentration was produced for each assay using dilutions of a 2mg/ml bovine serum albumin (BSA) solution in order to give concentrations of 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml and 25μg/ml in a final volume of 100μl. Each sample was then diluted to 1/100 and 1/200 (v/v) using PBS. Then, 100μl of the diluted sample was placed in a well of a 96-well micro-titre plate (Greiner). Each analysis was carried out in duplicate and 100μl of protein assay reagent was added to each well. The plate was incubated at 37°C for 2h to allow the reaction to occur. The absorbance of each sample was read at 595nm using a Dynex Technologies MRX microplate reader. The protein concentration of each sample was then calculated from the standard curve.

2.6.6 p44/42 MAPK (ERK) and SAPK/JNK Activity Assays

The p44/42 MAPK and SAPK/JNK assays were carried out using the appropriate non-radioactive kinase assay kits according to the protocol provided by the manufacturer (Cell Signalling Technology). Thus, the cells were washed with ice-cold PBS and ice-cold cell lysis buffer (1X) (provided in kit), supplemented with 1mM PMSF, was directly added to the cells (500μl for 250-300cm flask). Following 5min incubation on ice, the cells were scraped into the buffer. The cell extract was then sonicated for 20sec in ice and micro-centrifuged (10min at 10,000g) at 4°C. The supernatant was stored as cell lysate at -80°C.



To 200µl of cell lysate, 15µl of immobilised phospho-p42/44 MAPK (Thr202/Tyr204) antibody beads or 20µl of c-Jun fusion protein beads (provided in the kit) were added, and the sample was left to incubate overnight at 4°C with gentle rocking. The sample was then micro-centrifuged at 17,000g for 1min, and the resultant pellet was washed three times with 500µl of 1X cell lysis buffer, with micro-centrifugation at 17,000g for 1min between washes. The pellet was then washed three times with 500µl of 1X kinase buffer with micro-centrifugation at 17,000g for 1min between washes. Following the final wash, the pellet was resuspended in 50µl of 1X kinase buffer supplemented with 100µM of ATP (Elk-1 fusion protein was also added into the pellet for the p42/44 MAP kinase assay). The kinase mix was incubated at 30°C for 30min and the reaction terminated by the addition of 25µl of SDS-PAGE reducing gel loading buffer (Table 2.6). The sample was then boiled for 5min and loaded immediately onto a SDS-PAGE gel. SDS-PAGE and western blotting were carried out as described in sections 2.6.7 and 2.6.8 respectively. An antibody specific to the assay [anti-phospho-Elk1 (Ser383) for p44/p42 MAP kinase and anti-phospho-c-Jun (Ser63) for SAPK/JNK] was used for the immunodetection of the proteins (section 2.6.9, also see Table 2.8).

2.6.7 SDS-PAGE

SDS-PAGE was performed under reducing conditions following the method by Laemmli (1970), where separating gels and stacking gels were used. The gels were prepared from stock solutions (Table 2.6) as shown below in Table 2.7. Electrophoresis was carried out using the Mini-PROTEAN II slab electrophoresis cell from Bio-Rad Laboratories with the gel apparatus being assembled as described by the manufacturer (Bio-Rad Laboratories).

The separating gel was poured to within 3cm of the upper edge of the inner glass plate. Isopropanol was layered on top of the gel solution to exclude any air bubbles and the gel was allowed to polymerise. Once the gel had set, the isopropanol was

washed off with ddH_2O and the upper surface dried with Whatman 3MM paper. The stacking gel was then poured on top and the well-forming comb inserted. Following polymerisation of the stacking gel, the comb was removed and the wells were washed with ddH_2O. The gels were then placed in the electrophoresis tank and the upper and lower chambers were filled with running buffer containing 0.1% (w/v) SDS (Table 2.6).

Equal volume of protein samples prepared using the Laemmli sample buffer were mixed with an appropriate volume of bromophenol blue solution (Table 2.6), and then heated to 100°C for 7min before being loaded onto the gel. Equal amount of all the other protein samples were mixed with an appropriate volume of reducing gel loading buffer (Table 2.6) and then heated to 100°C for 3-5min. The samples were then cooled and loaded onto the gel. Rainbow protein size markers (10µl) (GE healthcare) were loaded into each gel (see Appendix I). The gel was subjected to electrophoresis at a constant voltage of 200V for 30-60min according to the size of protein. Gels were then used for western blotting (section 2.6.8).

Table 2.7 Composition of separating and stacking gels used for SDS-PAGE

Gel component	7.5% (w/v) Separating Gel	10% (w/v) Separating Gel	12.5% (w/v) Separating Gel	5% (w/v) Stacking Gel
Upper buffer	-	-	-	2.5ml
Lower buffer	2.5ml	2.5ml	2.5ml	-
Acrylamide: bisacrylamide (37.5:1)	1.875ml	2.5ml	3.125ml	1.25ml
ddH2O	5.625ml	5ml	4.26ml	6.25ml
10% (w/v) APS	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl

Abbreviations: APS, Ammonium persulphate; TEMED, N, N, N, N - tetra methyl ethylene diamine

2.6.8 Western blotting

Electrophoretic transfer of proteins was carried out using a Bio-Rad Trans Blot Electrophoretic transfer cell (Bio-Rad Laboratories). The transfer of proteins to PVDF membranes (Millipore) was carried out as described by the manufacturer. Briefly, the gel was removed from the glass plates and the stacking gel was cut away. The gel was then equilibrated by incubation in transfer buffer (Table 2.6) for 15min at room temperature. Whatman 3MM paper, activated PVDF membrane (soaked in methanol for 15sec) (both cut to the same size as the gel) and the sponge pads of the transfer apparatus were also pre-soaked in transfer buffer. The activated PVDF membrane was then placed on the gel and sandwiched between the Whatman 3MM paper and the sponge pads before being placed into the blotting cassette. The cassette was then subjected to electro-blotting in a tank containing transfer buffer at 4°C at a constant voltage of 15V overnight or at 150V for 1h. The proteins in the membrane were then subjected to immunodetection.

2.6.9 Immunodectection of proteins

The membrane was incubated in 20ml of blocking solution [1x TBS containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20] for 1h. The blocking solution was removed and the membrane washed three times (10min for each wash) in wash solution [1x TBS containing 0.1% (v/v) Tween-20]. The membrane was then incubated with the primary antibody, which was diluted in 1x TBS, containing 5% (w/v) skimmed milk or BSA and 0.1% (v/v) Tween-20, for 1h at room temperature or overnight at 4°C (Table 2.8). The primary antibody was removed and the membrane was washed as described above, and then immersed (10-20ml) in the appropriate secondary antibody diluted 1:10,000 (anti-STAT1, anti-β-actin and anti-nucleolin) or 1:2000 (anti-phospho-STAT1, anti-p44/42 MAP kinase, anti-phospho-p44/42 MAP kinase, anti-phospho-Elk1 and anti-phospho-c-Jun) in 1x TBS containing 5% (w/v) skimmed milk and 0.1% (v/v) Tween-20. Following incubation

for 1h at room temperature and series of washes detailed above, the detection of membrane bound reactive protein was carried out as described in the instructions supplied with the ECL detection kit (GE healthcare).

Table 2.8 Primary antibodies used for the immunodetection of the proteins

Primary antibody	Dilution in 1xTBS 0.1% (v/v) Tween 20	% (w/v) skimmed milk or BSA[1]	Incubation period[2]	Size (kDa)
Anti-STAT1	1:2000	5, skimmed milk	Overnight or 1h	84,91
Anti-phospho-STAT1 (Tyr701)	1:1000	5, BSA	Overnight	84,91
Anti-phospho-STAT1α (Ser727)	1:1000	5, BSA	Overnight	91
Anti-β-actin	1:10,000	5, skimmed milk	Overnight or 1h	42
Anti-phospho-Elk-1 (Ser383)	1:1000	5, BSA	Overnight	41*
Anti-phospho-c-Jun (Ser63)	1:1000	5, BSA	Overnight	35*
Nucleolin	1:5000	5, BSA	Overnight	110

1; BSA denotes bovine serum albumin.
2; Overnight and 1h incubations were carried out at 4°C and room temperature, respectively.
*; The size of the protein indicated in the table is the size of the product detected in kinase activity assay (according to Cell Signalling Technology), which are different from the endogenous protein.

2.7 Electrophoretic mobility shift assays (EMSA)

2.7.1 Stock solutions

Table 2.9 Stock solutions used in EMSA analysis

Solution	Composition
5× Binding buffer	50mM Tris-HCl (pH 8); 750mM KCl; 2.5mM EDTA; 0.5% (v/v) Triton X100; 62.5% (v/v) glycerol; 1mM DTT

2.7.2 Preparation of radiolabelled oligonucleotide probe

2.7.2.1 Annealing of oligonucleotides

The sequences of the oligonucleotides used for EMSA analysis are shown in Table 2.10. The forward and reverse sequences were designed to leave 5' overhangs, containing at least one G residue, following annealing to allow for complementary binding of [α^{32}P]-dCTP during radiolabelling.

The forward and reverse oligonucleotides (200ng of each) were incubated together at 100°C (10min) in the presence of a medium salt buffer (1x NEB Buffer 3) in a final volume of 100µl. The mixture was then allowed to cool to room temperature. Double stranded oligonucleotides prepared in this way were either radiolabelled immediately or stored at -20°C.

Table 2.10 Seqences of oligonucleotide probes for EMSA analysis

Probe	Annealed Oligonucleotide	Reference
MCP-GAS	5' GATCG CTT CCC TTT CCT ACT TCC TGG AAA 3' 3'GAAGGGAAAGGATGA AGGACC TTT AGGT 5'	(Matikainen et al., 2000)
STAT1 consensus sequence	5' CAT GTT ATG CAT ATT CCT GTA AGT G 3' 3' GTA CAA TAC GTA TAA GGACAT TCA C 5'	/
NFκB consensus sequence	5' GGA GTT GAG GGG ACT TTC CCA 3' 3' AA CTC CCC TGA AAGGGT CCG G 5'	/

2.7.2.2 Radiolabelling of double-stranded oligonucleotides

Radiolabelling was carried out using the reagents supplied in the Megaprime™ Labelling Kit (GE Healthcare) according to the manufacturer's protocol. The annealed double-stranded oligonucleotides (10ul) were incubated for 20min with 1x Labelling buffer, [α^{32}P]-dCTP (3µl) and Klenow DNA polymerase (2µl) in a final volume of 50µl at 37°C. Separation of the radiolabelled probe from unincorporated nucleotides was carried out using a Sephadex G50 nick column. The column was equilibrated with 1xTE buffer [10mM Tris-HCl (pH 7.5), 1mM EDTA] (9ml) and the reaction mix (50µl) loaded onto the column and eluted with 1x TE buffer (350µl), which was then discarded. The column was then further eluted with 1x TE (400µl) and the eluate collected and stored at -20°C.

2.7.3 DNA-protein binding reactions

The following components were added in order into the reaction:

Xµl of water to bring up the total volume of the mixture to 10µl;
2µl of poly-(dI-dC).poly-(dI-dC) (1µg/µl);
2µl 5X binding buffer (Table 2.9);
5µg nuclear extract;
3µl radiolabelled probe

The mixture was centrifuged briefly and incubated for 30min at room temperature, and then subjected to electrophoresis (section 2.7.5).

2.7.4 Antibody interference/supershift and competition binding studies

For antibody interference/supershift experiments, the antibody (2µg) was added to the nuclear extract and incubated on ice for 20min prior to the addition of the radiolabelled probe. In competition binding studies, the binding reaction mixture was incubated on ice for 20min with a 200-fold molar excess of unlabelled probe prior to the addition of the radiolabelled probe.

2.7.5 Electrophoresis of DNA-protein complexes

DNA-protein complexes were resolved by electrophoresis on 4-6% (w/v) non-denaturing polyacrylamide gels (Table 2.11). Electrophoresis was carried out for 3-4h at 150V or 12-18h at 35V (both at 4° C) using vertical gel apparatus (Scotlab) with 0.25x TBE as running buffer. Following electrophoresis, the gel was transferred to Whatmann 3MM paper and dried under vacuum using a Gel Dryer (Model 583, Bio-Rad) at 80° C for 1h. The dried gel was exposed to Kodak X-ray film in a light proof cassette (Genetic Research Instrumentation) at –80° C for varying exposure periods (12-72h). The X-ray films were developed using a Gevamatic 60 automatic developer (Agfa-Gevaert).

Table 2.11 Composition of non-denaturing polyacrylamide gels for EMSA analysis

Component	4% (w/v) Acrylamide	6% (w/v) Acrylamide
Acrylamide: Bisacrylamide (29:1)	5ml	7.5ml
10x TBE	2.5ml	2.5ml
ddH20	42.5ml	40ml
10% (w/v) APS	500µl	500µl
TEMED	50µl	50µl

2.7.6 Autoradiogaphy

The dried gel was placed in contact with Kodak X-ray film in a Hi-speed-X light proof cassette with an intensifying screen (Genetic Research Instrumentation) and stored at -80°C for varying exposure times (6-72h), depending on the strength of the signal. The film was developed in a Gevamatic 60 automatic developer (Agfa-Gevaert).

2.8 Leukocyte-endothelium adhesion assay

The CytoSelect™ Leukocyte-endothelium adhesion assay kit was provided by Cell BioLabs Inc. (San Diego, U.S.A.). The assay was performed according to the protocol of the manufacturer. Thus, 50,000 HUVEC cells/well were seeded in the Gelatin-coated 96-well plates and cultured until the endothelial cells formed a monolayer. The cells were then treated with effectors for an appropriate period of time. Monocytes (THP-1 or U937 cell line) were prepared in cell suspension at $1x10^6$ cells/ml in serum free media (SFM) and then incubated with LeukoTracker (1x) (provided in kit) for 60min at 37°C in a cell culture incubator. Following incubation, the cells were washed twice with SFM and finally resuspend in SFM at $0.5x10^6$cells/ml. Then, 200µl of LeukoTracker-labelled monocytes were added into each well and allowed to attach to HUVEC monolayer for 1h. After washing 3 times, the adherent cells were lysed with 1x lysis buffer (provided in kit) and quantified by fluorescence measurement which was performed on FluoStar Optima (BMG LABTECH, Aylesbury, UK) at 485nm/520nm. The background values using the 1x lysis buffer (100µl) alone were subtracted from the sample values.

2.9 Densitometric analysis of the data

The intensity of bands from agarose gel images and immunoblots were analysed using GeneTools™ (Syngene) software as described by Harvey et al (2007).

2.10 Statistical analysis of the data

To assess data for statistical significance a standard student's *t* test was carried out, the details of which are given in the Appendix III.

CHAPTER THREE:

REGULATION OF GENE EXPRESSION AND STAT1 PHOSPHORYLATION BY IFN-γ

Chapter 3: Regulation of gene expression and STAT1 phosphorylation by IFN-γ in human macrophages

3.1 Introduction

As described in General Introduction, IFN-γ is known to regulate the expression of numerous genes in macrophages, many of which play significant roles in the progression of atherosclerosis (section 1.5). Studying the signalling mechanism underlying the IFN-γ-regulated expression of such genes could potentially identify novel therapeutic targets for the treatment of this disease. The JAK–STAT pathway is known as a key signalling cascade mediating IFN-γ-regulated gene transcription. In particular, STAT1 phosphorylation at tyrosine 701 and serine 727 plays critical roles in the regulation of gene expression and cellular function by IFN-γ (section 1.6).

3.1.1 Specific aims

Previous work in our laboratory, most of which was carried out in the mouse macrophage J774.2 cell line, has led to great successes in unravelling the mechanisms underlying the IFN-γ-regulated transcription of key genes implicated in inflammation and atherosclerosis. The studies have identified potentially key roles for CK2 and the PI3K pathways (Hughes et al., 2002; Mead et al., 2003; Harvey et al., 2007; Harris et al., 2008). In the light of these findings, we decided to further explore the pathways by which IFN-γ regulates gene expression in human macrophages. In order to achieve this, it was first decided to validate the use of a human macrophage cell line as a model system for future work.

3.1.2 Experimental strategy

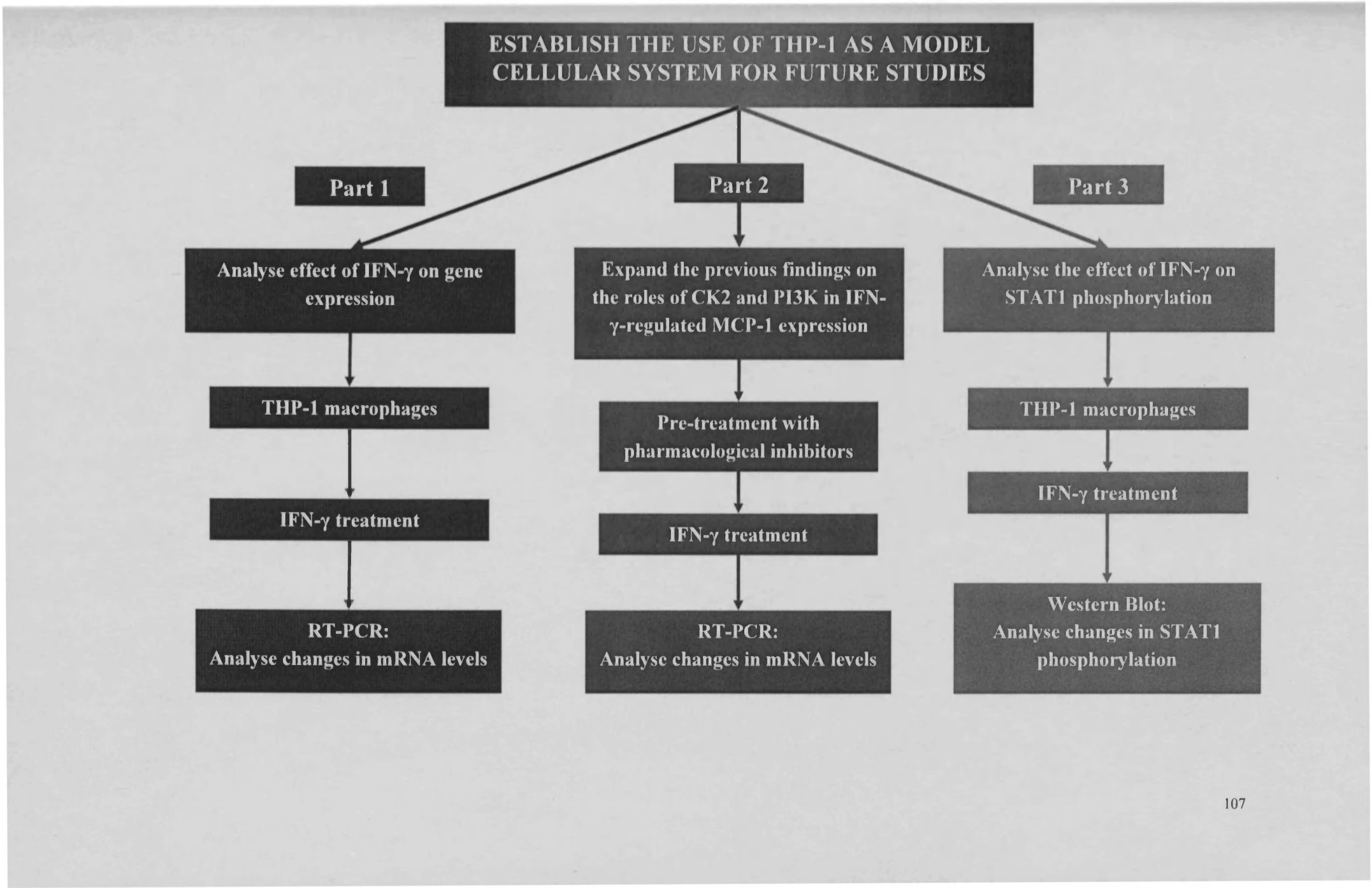
The human monocytic cell line THP-1 was selected as a model system because it has been employed extensively to investigate the cytokine-mediated regulation of gene expression relevant to the pathogenesis of atherosclerosis (Hsu et al., 1996; Kang et al., 2006; Singh and Ramji, 2006; Imaizumi et al., 2007). In many of these studies, THP-1 cells were stimulated with phorbol esters to initiate their differentiation into macrophage-like cells, which mimic native monocyte-derived macrophages (Kosaka et al., 2001; Imaizumi et al., 2007; Kang et al., 2007). It has been claimed that compared to other human myeloid cell lines, such as HL-60 and U937, differentiated THP-1 cells behave more like native monocyte-derived macrophages (Auwerx, 1991). Indeed, numerous responses and underlying mechanisms of actions in this cell line have been found to be conserved with primary cultures (Shiratsuchi and Basson, 2005; Singh and Ramji, 2006; Stephenson et al., 2006; Harvey et al., 2007). In contrast to human primary monocyte-derived macrophages with potential donor-specific variations, the THP-1 cell line provides a more homogeneous system and also allows the extraction of a greater amount of RNA or protein. The PMA-differentiated THP-1 cell line has been used successfully to model macrophage responses to various mediators in our laboratory, producing data that has subsequently been replicated in primary cells and other macrophage cell lines (Hughes et al., 2002; Mead et al., 2003; Greenow, 2004; Singh and Ramji, 2006; Harvey et al., 2007; Huwait, 2008).

In order to establish the routine use of THP-1 cells for future studies, it was decided initially to analyse the effect of IFN-γ on the expression of several model genes that have previously been employed in our laboratory to investigate the action of this cytokine in other macrophage cell lines and primary cultures (Harvey et al., 2007; Harris et al., 2008). The genes are discussed below in detail. It was hoped that such studies would validate previous findings and identify the optimal concentration and

time point for cytokine treatment for each gene studied. Once this had been established, the next step was to expand the previous findings on the potentially important roles for CK2 and PI3K in IFN-γ signalling identified using the mouse system to human macrophages. This would involve analysis of the effect of pharmacological inhibitors of these pathways on the induction of MCP-1 expression by IFN-γ. Finally, it was decided to investigate the effect of IFN-γ on STAT1 phosphorylation at serine 727 and tyrosine 701 in THP-1 macrophages given that this is involved in the activation of this transcription factor, and thereby the regulation of numerous downstream genes (section 1.6). The information gained from these experiments would then be used for future investigation of the mechanisms underlying the STAT1-mediated IFN-γ signalling in human macrophages. The overall experimental strategy employed for the studies described in this chapter is outlined in the flow chart shown in Figure 3.1.

Figure 3.1 Experimental strategy

The aims of the studies presented in this chapter were to establish the use of THP-1 as a model cellular system for future studies. Part 1 of the experimental strategy was to analyse the effect of IFN-γ on the expression of each model gene. This would lead to the identification of the optimal concentration and time point for cytokine treatment for each gene studied. The aim of part 2 was to confirm and to expand on the previous finding of crucial roles for CK2 and PI3K in the IFN-γ-regulated expression of MCP-1 to THP-1 macrophages using pharmacological inhibitors. Finally, part 3 of the experimental strategy was to establish the use of THP-1 in investigating the regulation of STAT1 phosphorylation by IFN-γ using western blot analysis.


ESTABLISH THE USE OF THP-1 AS A MODEL CELLULAR SYSTEM FOR FUTURE STUDIES
Part 1
Part 2
Part 3
Analyse effect of IFN-γ on gene expression
THP-1 macrophages
IFN-γ treatment
RT-PCR: Analyse changes in mRNA levels
Expand the previous findings on the roles of CK2 and PI3K in IFN-γ-regulated MCP-1 expression
Pre-treatment with pharmacological inhibitors
IFN-γ treatment
RT-PCR: Analyse changes in mRNA levels
Analyse the effect of IFN-γ on STAT1 phosphorylation
THP-1 macrophages
IFN-γ treatment
Western Blot: Analyse changes in STAT1 phosphorylation

3.1.3 CK2

CK2 is a ubiquitous, constitutively active protein kinase, typically present as a tetrameric holoenzyme complex of two catalytic subunits (α and/or α') and two regulatory β subunits (Litchfield, 2003; Singh and Ramji, 2008). A third catalytic subunit, α'' has also been discovered in hepatocytes (Shi et al., 2001). CK2 has traditionally been classified as a serine/threonine protein kinase. However, there are reports showing it as also being capable of phosphorylating tyrosine residues, suggesting that CK2 may be a dual-specificity kinase (Marin et al., 1999; Litchfield, 2003). CK2 is known to catalyse the phosphorylation of a broad range of substrates, including signalling molecules, transcription factors and proteins implicated in RNA synthesis and translation (Meggio and Pinna, 2003 and references therein). In particular, CK2 has been recognized to modulate several key transcription factors [e.g. nuclear factor-κB (NF-κB), STAT1, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), c-Jun, c-Fos] that have been implicated in inflammatory responses and associated diseases (Singh and Ramji, 2008). A potential key role for CK2 in the IFN-γ-regulated expression of several key genes implicated in atherosclerosis has also been identified, mainly through studies using the mouse macrophage J774.2 cell line (Mead et al., 2003; Harvey et al., 2007).

3.1.4 PI3Ks

PI3Ks are a family of related lipid kinases which phosphorylate 3-OH position of the inositol ring of phosphoinositides. They have been linked to a diverse group of physiological processes, including cell growth, proliferation, differentiation, and migration. PI3K-dependent signalling has been implicated in the pathogenesis of a number of disorders, including cancer, allergy, diabetes, cardiovascular disease and chronic inflammation (Rameh and Cantley, 1999; Krasilnikov, 2000; Fruman, 2004). For example, the activation of PI3K, along with its downstream effector Akt (also called protein kinase B), by oxLDL and inflammatory chemokines observed in

inflammatory cells is absent in macrophages derived from mice that are deficient in the PI3K p110γ catalytic subunit. In addition, the aggressive development of atherosclerotic plaques in apoE-/- mice was significantly reduced in the absence of p110γ (Chang et al., 2007). These results strongly demonstrate that the PI3K pathway plays a significant role in the development of atherosclerosis and thus provides a promising target for the treatment of this disease.

A number of studies have shown that PI3Ks play important roles in the IFN-γ-mediated gene expression and cellular responses, and appear to exert their function via activation of the major downstream effector Akt (Nguyen et al., 2001; Kristof et al., 2003; Navarro et al., 2003; Choudhury, 2004; Hwang et al., 2004; Rosner et al., 2006; Venkatesan et al., 2006). In addition, other studies have shown that in certain cellular systems, IFN-γ induces STAT1 phosphorylation at Ser727 and this is associated with transcriptional activation in a PI3K-Akt-dependent manner (Nguyen et al., 2001; Kristof et al., 2003; Choudhury, 2004; Venkatesan et al., 2006). PI3K appears to be dispensable for Tyr701 phosphorylation as shown by most of these studies. However, Hwang, et al. (2004) have found that the PI3K inhibitor LY294002 attenuates the IFN-γ-stimulated phosphorylation at this site in microglial cells.

Although most of the studies suggest that the PI3K pathway regulates IFN-γ responses via modulation of STAT1 activation, a PI3K-dependent, but STAT1-independent pathway, has also been demonstrated (Navarro et al., 2003). For example, Nguyen et al. (2001) have observed the activation of PI3K/Akt in response to IFN-γ in JAK1- or JAK2-deficient cells, where STAT1 phosphorylation was abolished. It is therefore possible that PI3K takes part in IFN-γ signalling through both STAT1-dependent and JAK-STAT-independent manner.

3.1.5 Genes selected for study

To investigate the IFN-γ signalling pathways in the regulation of macrophage gene expression, our laboratory has employed a range of model genes, which are all known to exert critical, but distinct, functions in inflammation and atherosclerosis.

3.1.5.1 MCP-1

Amongst the genes that have been previously demonstrated to be regulated by CK2 and the PI3K pathway in response to IFN-γ is the key chemokine MCP-1(Harvey et al., 2007), the pro-atherogenic role of which has been detailed before (section 1.4.2.4). Because of such an important role, investigation of the mechanisms underlying the regulation of this gene will not only enhance our understanding of the molecular basis of atherosclerosis, but could also ultimately lead to the identification of potentially novel avenues for the treatment of this disease.

The expression of MCP-1 has been reported to be induced by IFN-γ at the transcriptional level in a variety of cell types. Such regulation by IFN-γ appears to be controlled predominantly by the proximal promoter region (-213/+1), which contains a number of putative consensus regulatory sequences, including the GAS site, GC-box, AP-1 binding sites (i.e. TPA-responsive element (TRE)), NF-κB binding site and possibly C/EBP binding sites (Figure 3.2). The interaction of transcription factors with these binding sites have been demonstrated in different cell lines and in response to various mediators that are known to regulate MCP-1 gene transcription (Valente et al., 1998; Zhou et al., 1998; Lim and Garzino-Demo, 2000; Ping et al., 2000; Zhou et al., 2001). For example, studies in astrocytes and osteoblastic cells have demonstrated the presence of a functional STAT1-binding site (GAS site) that is responsible for the IFN-γ-inducible promoter activation (Valente et al., 1998; Zhou et al., 1998; Zhou et al., 2001). It should, however, be noted that a study on gene expression in STAT1-null bone marrow-derived macrophages (BMMs) showed the induction of MCP-1 by IFN-γ in both wild type

and STAT1-deficient cells (Gil et al., 2001; Ramana et al., 2001). The precise reason(s) for this is unclear but could be because of the potential existence of STAT1-independent pathways or the high expression level of STAT3, which can also bind to GAS sites with lower affinity, that has been found in STAT1-deficient cells (Ramana et al., 2005).

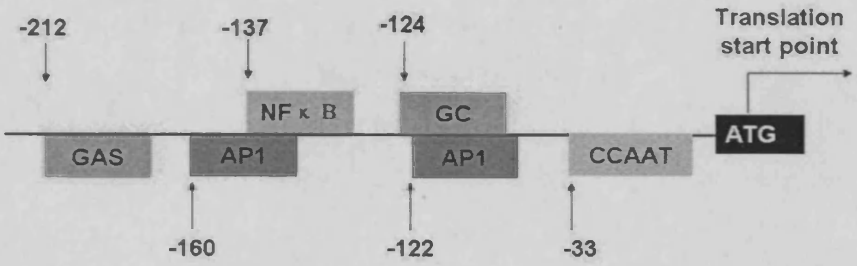


Figure 3.2 Schematic representation of the proximal *cis*-regulatory region of the MCP-1 gene promoter

The region from -212 to the ATG translation start codon is indicated with the putative binding sites of key transcription factors (boxed). Box with orange colours represents the binding sites that have been found to be crucial for the IFN-γ-inducible MCP-1 gene expression.

3.1.5.2 SOCS-1

SOCS-1 is a member of the SOCS protein family, which as the name suggests, act as negative regulators of the JAK-STAT pathway activated by a range of cytokines (Nicola and Greenhalgh, 2000; Kile and Alexander, 2001). SOCS-1 inhibits the JAK-STAT pathway by acting as a pseudosubstrate of JAK2 (Giordanetto and Kroemer, 2003), and can also facilitate the degradation of JAKs (Valentino and Pierre, 2006). The expression of SOCS-1 is at low levels in unstimulated cells, but is induced in different cell types by many inflammatory cytokines, including IFN-γ. SOCS-1 in turn suppresses cytokine signalling in a classical negative feedback pathway (Nicola and Greenhalgh, 2000; Krebs and Hilton, 2001; Alexander and

Hilton, 2004). SOCS-1-/- mice develop a neonatal lethal phenotype due to a complex inflammatory disease (Alexander et al., 1999). This pathology is thought to be caused by excessive responses to IFN-γ as SOCS-1/IFN-γ double knock-out mice do not suffer from such condition (Alexander et al., 1999). A number of other transgenic animal studies have indicated that the expression of SOCS-1 can reduce sustained IFN-γ signalling associated with chronic inflammatory diseases (Egan et al., 2003; Chen et al., 2004; Fujimoto et al., 2004). SOCS-1 has also been recently found in atherosclerotic lesions in apoE-/- mice (Tang et al., 2005) and is now recognised as a potential target for therapeutic intervention of this disease (Tang and Raines, 2005). Ramana et al. (2001) demonstrated that the expression of SOCS-1 is induced by IFN-γ in wild type cells but not in STAT1-null BMMs, thereby suggesting that the IFN-γ-inducible expression of SOCS-1 is STAT1-dependent.

3.1.5.3 Lipoprotein lipase (LPL)

The LPL enzyme expressed by macrophages is known to exert a pro-atherogenic role. The enzyme catalyses the hydrolysis of the triacylglycerol component of circulating chylomicrons and very low density lipoproteins (VLDL). As triacylglycerols are removed by hydrolysis, chylomicrons shrink in size and become chylomicron remnants that are readily taken up by macrophages (Mead and Ramji, 2002). In addition, the free fatty acids produced by the action of LPL can be re-esterfied by macrophages. Together, these processes lead to the increase of cholesteryl esters within macrophages. The hydrolysis of VLDL also produces LDL, the modification of which, such as oxidation by free radicals in intimal space increases their rate of uptake into macrophages (Mead et al., 2002). oxLDL is one of the major contributors to the development of atherosclerotic lesion (section 1.4.2.1). Moreover, because of the existence of two separate domains for binding to lipoproteins and the cell surface, the enzyme also possesses a non-catalytic bridging action that causes the accumulation of lipoproteins at the cell surface, and thereby

their subsequent uptake by the cells (Mead et al., 2002; Mead and Ramji, 2002). Both the catalytic and the non-catalytic actions of LPL contribute to the transformation of macrophages into foam cells (Palmer et al., 2005; Bravo and Napolitano, 2007). In support for a pro-atherogenic role, decreased diet-induced atherosclerotic lesion formation was observed in chimeric mice that are deficient for macrophage LPL expression, thereby suggesting that LPL deficiency in macrophages has a protective effect against a highly atherogenic lipoprotein profile (Babaev et al., 1999; Babaev et al., 2000; Van Eck et al., 2000). The expression of LPL has been shown to be inhibited by IFN-γ in human macrophages (Jonasson et al., 1990). Previous work in our laboratory has shown that CK2 and PI3K signalling pathways play important roles in the IFN-γ-mediated inhibition of macrophage LPL gene transcription through the regulation of Sp1/Sp3 binding (Hughes et al., 2002; Harris et al., 2008).

3.2 Results

3.2.1 The effect of IFN-γ on the expression of MCP-1, SOCS-1 and LPL genes

The effect of IFN-γ on the expression of MCP-1, SOCS-1 and LPL genes in human THP-1 macrophages over a 24h time course was analysed using semi-quantitative RT-PCR (section 2.4.2). Following differentiation, THP-1 cells were incubated with or without IFN-γ for periods of 1, 3, 6, 12 and 24h, after which total RNA was extracted. The concentration of IFN-γ used here was exactly the same as that employed for previous studies in the J774.2 cell line (Harvey et al., 2007). The integrity of total RNA was determined by resolving an aliquot on a 1% agarose gel. As shown in Figure 3.3A, the extracted RNA was of high quality as the relative density of 28S rRNA band was approximately twice compared to that for 18S rRNA band for each sample. Such high quality RNA was consistently obtained for all studies carried out in this thesis and hence the data are not shown in all cases to avoid repetition. Semi-quantitative RT-PCR was carried out for each of the genes using the primers and conditions detailed in Tables 2.3 and 2.4 respectively. The optimal RT-PCR condition for MCP-1 and LPL has been previously determined in our laboratory (Harvey, 2006; Harvey et al., 2007; Huwait, 2008). The condition for SOCS-1 and 28S rRNA was optimised as a part of the research carried out during the course of this thesis (data not shown).

Figure 3.3B shows the PCR products for MCP-1, SOCS-1 and LPL as fractionated by agarose gel electrophoresis. The absence of PCR product in the 'no reverse transcriptase' (-RT) reaction indicates that the amplification products were not derived from any contamination of the samples with genomic DNA. The size of each amplification product was determined from DNA molecular weight markers (Appendix I) that were analysed alongside. The images shown in Figure 3.3B are representative of three independent experiments. Although the technique is semi-

quantitative in nature, an indication of relative changes can be gauged more easily by densitometric analysis of the amplification products. Densitometric analysis (section 2.9) was therefore carried out and the relative intensity of the bands at each time point was calculated in relation to the intensity of a PCR product band for a control gene (28S rRNA) to ensure equal cDNA input. The data was normalised to the 1h untreated control and expressed as a fold change relative to this (Figure 3.3C). Student's *t* test (Appendix III) was carried out for this data and the changes in expression were significant to the degree indicated. It is worth noting that there was no significant change in constitutive expression (i.e. in the absence of IFN-γ) of all genes analysed throughout the time course. Untreated sample at 1h was therefore chosen as constitutive control to allow indication of whether there was any change in constitutive and IFN-γ treated samples.

MCP-1 mRNA was present at undetectable levels in untreated cells (Figure 3.3). However, the mRNA levels increased continuously after IFN-γ treatment peaking at 12h but declining at 24h treatment. The increase at all time points observed was statistically significant in relation to untreated 1h control. Similar to MCP-1, SOCS-1 had low expression without IFN-γ treatment and this was induced by the cytokine to reach maximal levels at the 12h point. At 24h post-treatment, the expression of SOCS-1 decreased back to the similar level as that observed in untreated samples. Unlike these two genes, LPL was expressed at high levels throughout the 24h incubation period without IFN-γ treatment. In IFN-γ-treated cells, the mRNA levels started declining significantly at 3h and this continued throughout the 24h incubation period. A comparison of the mRNA levels at each of the time points for untreated control samples confirmed that the changes in expression were due to the effect of the cytokine and not the status of the cells at each of the time point.

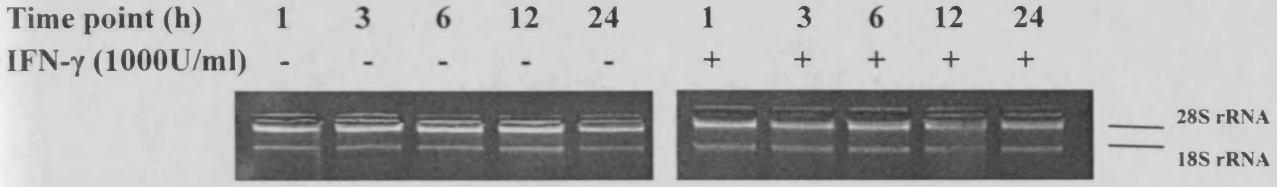
The constant 28S/18S rRNA levels and the expression of 28S rRNA as judged by RT-PCR, suggest that IFN-γ does not have a global effect on cellular gene

transcription. In addition, a trypan blue exclusion assay (section 2.3.12.1) was carried out and showed that there were a negligible proportion of cells dying throughout the time course (Table 3.1). Overall, therefore , these results suggest that the changes in gene expression observed for these IFN-γ-regulated genes was not due to a toxic effect of the cytokine on the cells.

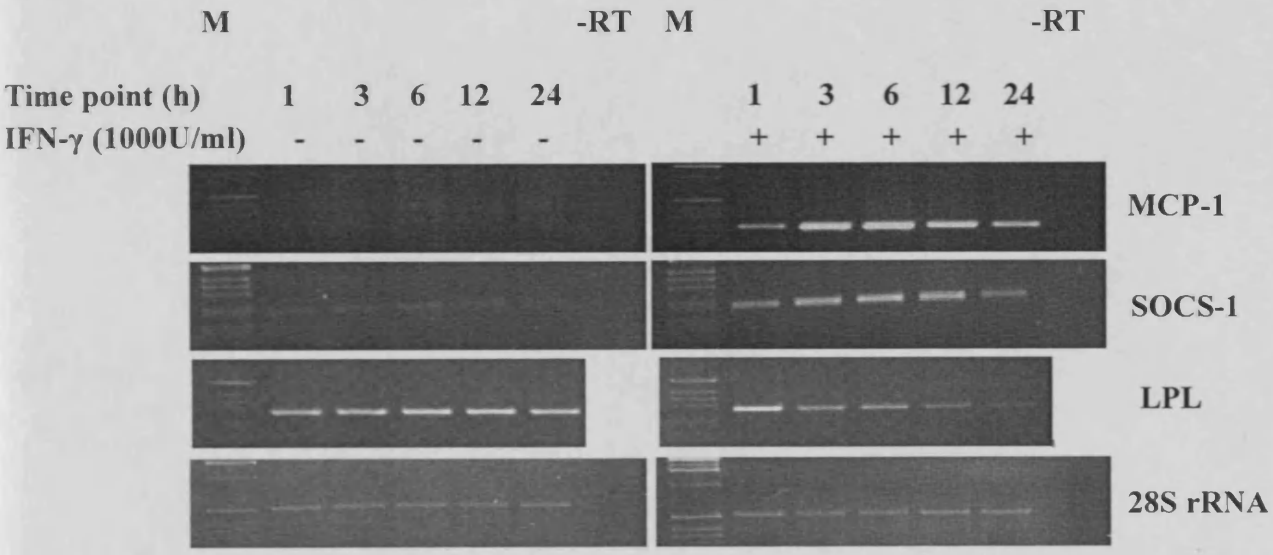
Figure 3.3 Time-dependent effect of IFN-γ on MCP-1, SOCS-1 and LPL mRNA expression

PMA-differentiated THP-1 macrophages were exposed to IFN-γ (1000U/ml) or left untreated for the indicated period of time. Total RNA was isolated and size-fractionated by electrophoresis on a 1% agarose gel (A). RNA was subjected to RT-PCR using primers against MCP-1, SOCS-1, LPL and 28S rRNA as shown. The amplification products were analysed by agarose gel electrophoresis. The images shown are representative of three independent experiments (B). M denotes the 1kb DNA ladder (Appendix I) and –RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (RNA from cells incubated for 1h with IFN-γ were used and this has only been carried out for MCP-1, SOCS-1 and 28S rRNA). Panel C shows the fold changes (mean ±SD) in the expression of the indicated gene normalised to the expression of 28S rRNA as determined by densitometric analysis from three independent experimental series (1h untreated control assigned as 1). Green and blue bars represent the untreated and IFN-γ-treated samples respectively. Statistical comparisons were carried out using the Student's *t* test between the 1h untreated control and treated samples at each of time point (*, $P<0.05$; ***, $P<0.001$; N/S, not significant).

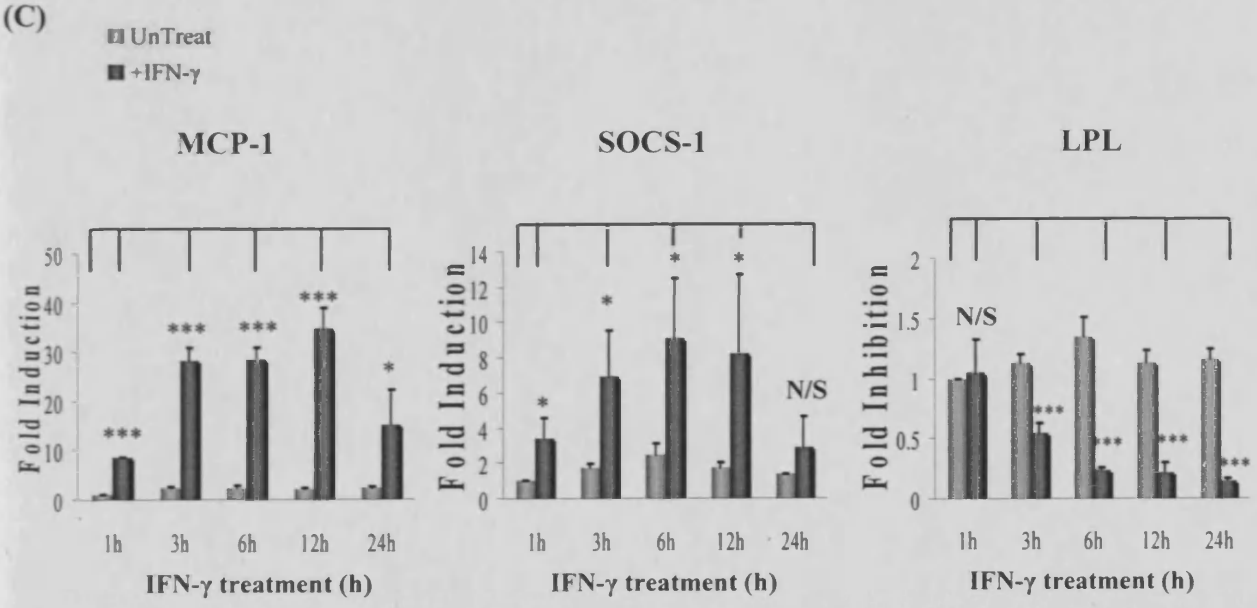
(A)


Time point (h) 1 3 6 12 24 1 3 6 12 24
IFN-γ (1000U/ml) - - - - - + + + + +
28S rRNA
18S rRNA

(B)


M -RT M -RT
Time point (h) 1 3 6 12 24 1 3 6 12 24
IFN-γ (1000U/ml) - - - - - + + + + +
MCP-1
SOCS-1
LPL
28S rRNA

(C)


UnTreat
+IFN-γ
MCP-1
SOCS-1
LPL
Fold Induction
Fold Inhibition
N/S
1h 3h 6h 12h 24h
IFN-γ treatment (h)

	Untreated	Treated with IFN-γ				
		1h	3h	6h	12h	24h
THP-1 viability (%)	100	100	98	98	98	96

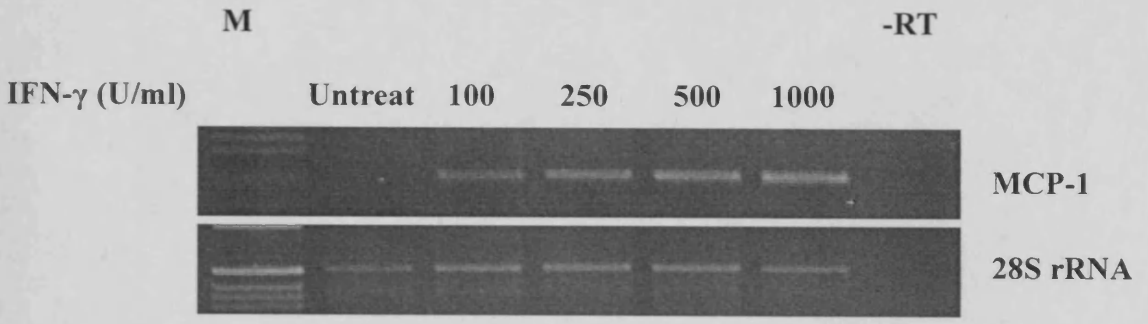
Table 3.1 Assessment of toxicity of IFN-γ on THP-1 macrophages over a time course

PMA-differentiated THP-1 cells were either left untreated or exposed to IFN-γ (1000U/ml) for the indicated period of time. The viability of the cells was than assessed using the trypan blue exclusion assay, which detects dead cells taking up the trypan blue stain. The average percentage viability of cells at each time point from two independent experiments is shown.

3.2.2 Concentration-dependent effect of IFN-γ on the expression of MCP-1

The experiments shown in Figure 3.3 used only a single concentration of IFN-γ (1000U/ml). Dose-response experiments were therefore carried out using MCP-1 as a model gene in order to determine an optimal concentration of this cytokine that is required to produce a maximal response. The 3h time point was chosen because it is the earliest time point where a marked induction of MCP-1 mRNA expression was observed. Thus, THP-1 macrophages were either left untreated or incubated for 3h with four different concentrations of IFN-γ (100, 250, 500 and 1000U). Total RNA was then extracted for semi-quantitative RT-PCR analysis. Figure 3.4 shows that the expression of MCP-1 mRNA was induced maximally at a concentration of 1000U/ml of IFN-γ. As this concentration had negligible effect on the viability of THP-1 macrophages at the 3h time point, it was selected for all future studies with THP-1 cells presented in this thesis.

(A)



(B)

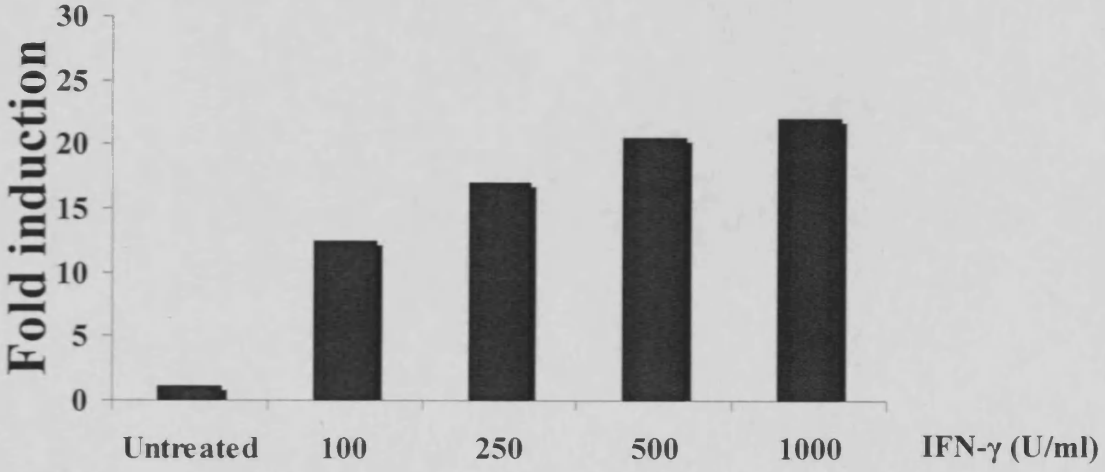


Figure 3.4 Concentration-dependent effects of IFN-γ on MCP-1 mRNA expression

PMA-differentiated THP-1 macrophages were either left untreated or exposed for 3h with the indicated concentration of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1 and 28S rRNA. The amplification products were analysed by agarose gel electrophoresis. The images shown are representative of two independent experiments (A). M denotes the 1kb DNA ladder (Appendix I) and –RT denotes a reaction in which no reverse transcriptase was included during the cDNA synthesis step (RNA from cells treated with 1000U/ml of IFN-γ was used). Panel B shows the average fold induction in the expression of MCP-1 normalised to the expression of 28s rRNA as determined by densitometric analysis (untreated control assigned as 1) from two independent experimental series.

3.2.3 Effect of a pharmacological inhibitor of CK2 on the IFN-γ-induced MCP-1 expression

Work in our laboratory has demonstrated novel roles for CK2 and PI3K in the IFN-γ-mediated regulation of macrophage gene expression implicated in atherosclerosis. These studies were mainly carried out using the mouse macrophage J774.2 cell line. It was therefore decided to replicate key experiments in THP-1 macrophages. MCP-1 was selected as a model gene because of the marked induction in its expression by IFN-γ at 3h (Figure 3.3). The specific pharmacological inhibitor of CK2 (apigenin) was used at concentration that is widely used in the published literature (Table 2.2) and in our laboratory (Ali, 2007; Harvey et al., 2007; Huwait, 2008).

PMA-differentiated THP-1 macrophages were pre-treated with apigenin (20μM) or DMSO as a vehicle control (section 2.3.8). They were then cultured in the presence or the absence of IFN-γ for 3h. RNA was extracted and its quality was determined by resolving a small aliquot by agarose gel electrophoresis (Figure 3.5A). RT-PCR analysis was then carried out using primers against MCP-1 and GAPDH (Figure 3.5B). GAPDH is another routinely used constitutive control gene in our laboratory and its optimal RT-PCR conditions have been previously determined in our laboratory (N. Singh, personal communication) and are detailed in Table 2.4. GAPDH was employed here because of unanticipated sudden problems with 28S rRNA RT-PCR when these experiments were carried out. As expected, IFN-γ increased the expression of MCP-1 mRNA and this was attenuated in the presence of apigenin (Figure 3.5C). Thus, similar to mouse J774.2 macrophages, CK2 is also involved in the IFN-γ-mediated regulation of MCP-1 expression in THP-1 macrophages.

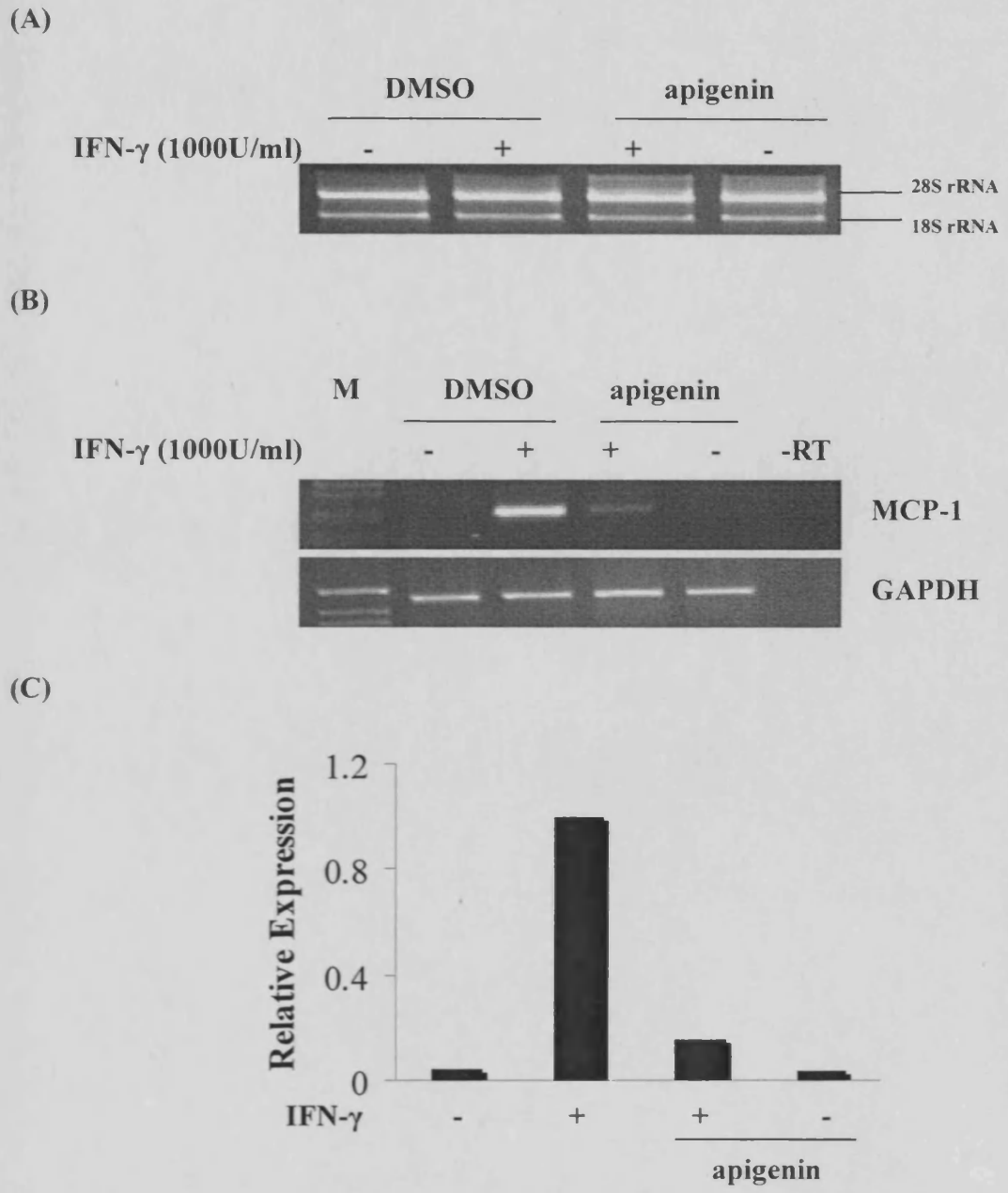


Figure 3.5 Effect of apigenin on the regulation of MCP-1 mRNA expression by IFN-γ

PMA-differentiated THP-1 macrophages were pre-treated for 1h with apigenin (20μM) or DMSO as a vehicle control. The cells were then cultured in the presence or the absence of IFN-γ for 3h. Total RNA was isolated and size-fractionated by agarose gel electrophoresis (A). RNA was subjected to RT-PCR using primers against MCP-1 and GAPDH. The amplification products were analysed by agarose gel electrophoresis and the images shown are representative of two independent experiments (B). M denotes the 1kb DNA ladder (Appendix I). –RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (RNA from cells incubated with DMSO and IFN-γ were used). Panel C shows the average relative expression of MCP-1 normalised to the expression of GAPDH (IFN-γ-treated, DMSO control assigned as 1) from two independent experiments.

The study detailed above used only a single concentration of apigenin (20μM). It was therefore decided to assess whether the effect of this compound on MCP-1 mRNA expression occurred in a concentration dependent manner. Therefore, THP-1 macrophages were pre-treated with various concentrations of the inhibitor or DMSO as a vehicle control (the volume of DMSO corresponding to the highest concentration of apigenin (20μM) was used). The cells were then either left untreated or incubated with IFN-γ for 3h. The expression of MCP-1 and 28S rRNA was determined by semi-quantitative RT-PCR.

As shown in Figure 3.6, apigenin significantly attenuated the IFN-γ-mediated induction of MCP-1 expression in a concentration-dependent manner. In contrast, the expression of the constitutive control, 28S rRNA was not affected by the inhibitor, thereby indicating that the inhibitory effect of apigenin on the IFN-γ-mediated induction of MCP-1 expression was not a result of global action of the inhibitor on cellular gene expression. In addition, a trypan blue exclusion assay (section 2.3.12.1) was carried out to confirm that the observed changes in expression were not because of a toxic effect of the inhibitor. The results showed over 86% cellular viability following treatment of cells with apigenin at 20μM and the cytokine (Table 3.2). Together, these results further support the hypothesis that CK2 plays a potentially important role in the regulation of MCP-1 expression in response to IFN-γ stimulation in human macrophages.

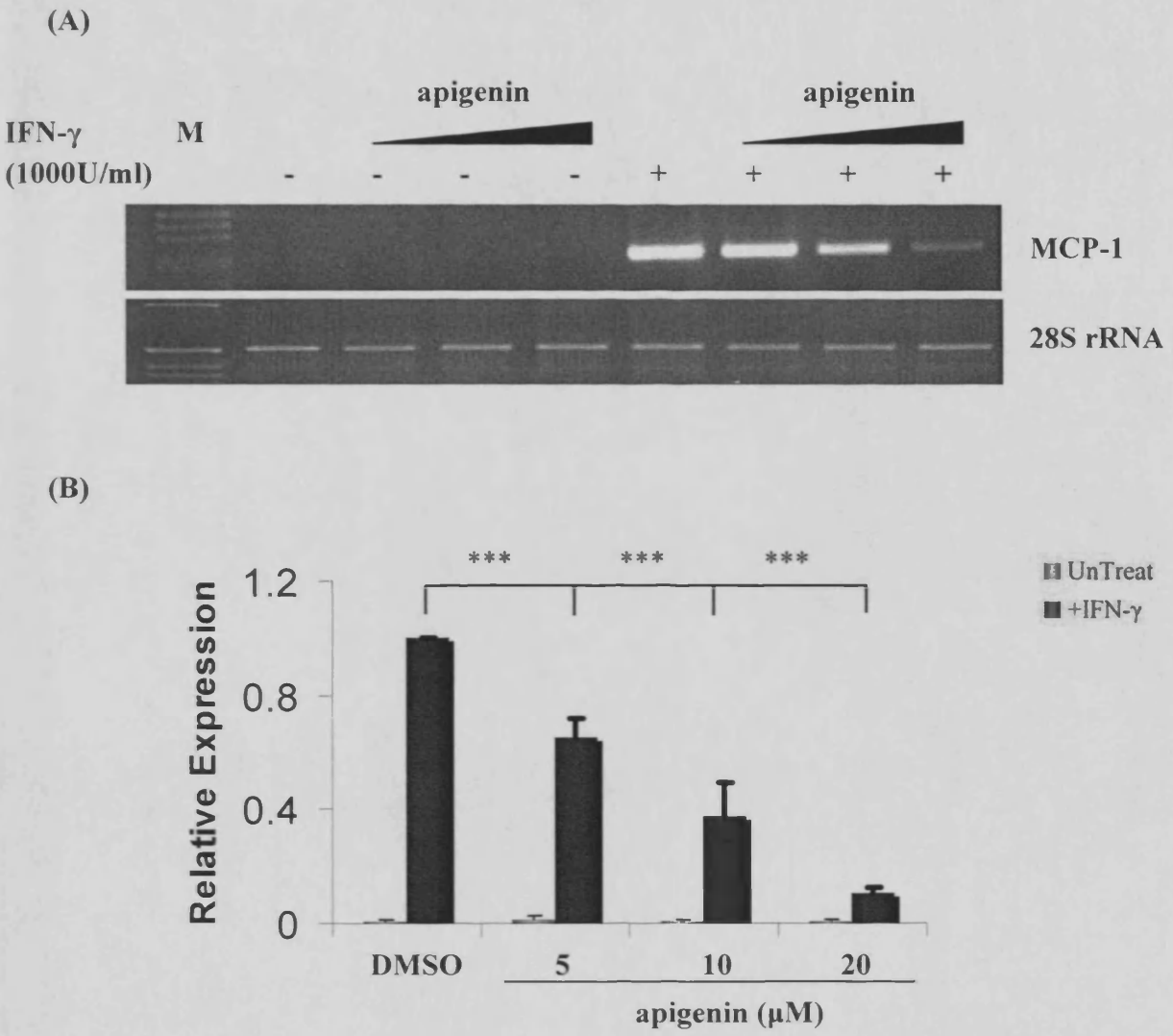


Figure 3.6 Concentration-dependent effect of apigenin on the regulation of MCP-1 mRNA expression by IFN-γ

PMA-differentiated THP-1 macrophages were pre-treated for 1h with apigenin (5, 10 and 20μM) or DMSO as a vehicle control. The cells were then cultured in the presence or the absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1 and 28S rRNA as shown. The amplification products were analysed by agarose gel electrophoresis and the images are representative of three independent experiments (A). M denotes the 1kb DNA ladder (Appendix I). Panel B shows the relative expression (mean ±SD) of MCP-1 normalised to the expression of 28S rRNA (IFN-γ-treated, DMSO control assigned as 1) from three independent experiments. Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with apigenin and IFN-γ (***, $P<0.001$).

	DMSO	DMSO	apigenin	LY294002
IFN-γ	-	+	+	+
THP-1 viability (%)	100	100	86	92

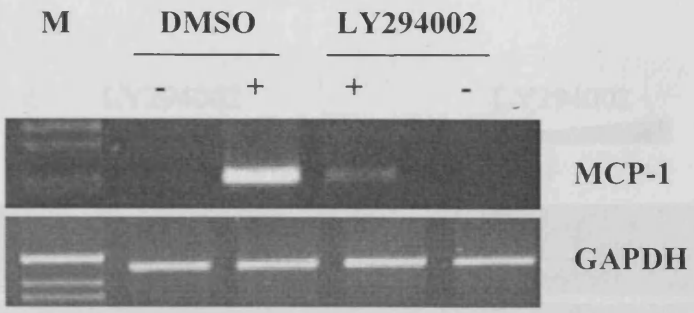
Table 3.2 Assessment of toxicity of apigenin and LY294002 with IFN-γ on THP-1 macrophages at the 3h time point

PMA-differentiated THP-1 cells were pre-treated for 1h with apigenin (20μM) or LY294002 (20μM). The cells were subsequently either left untreated or exposed to IFN-γ (1000U/ml) for 3h. The viability of the cells was than assessed using the trypan blue exclusion assay, which detects dead cells taking up the trypan blue stain. The data was obtained from a single experiment.

3.2.4 Effect of the pharmacological inhibitor of PI3K on the IFN-γ-induced MCP-1 expression

In addition to CK2, PI3K has been demonstrated to play a critical role in the regulation of gene expression by IFN-γ in our laboratory using mainly mouse macrophages (Harvey et al., 2007). It was therefore decided to next investigate the effect of the PI3K inhibitor, LY294002, on the IFN-γ-mediated regulation of MCP-1 expression in human THP-1 macrophages. The experiment was carried out exactly as described in section 3.2.3, except that the cells were treated with LY294002 instead of apigenin. Semi-quantitative RT-PCR of MCP-1 and GAPDH was carried out and the results are presented in Figure 3.7 and 3.8. The results show that LY294002 significantly attenuates the IFN-γ-induced expression of MCP-1 in a concentration-dependent manner. In addition, the trypan blue assay shows that treatment of THP-1 macrophages with LY294002 at 20μM resulted in over 90% cellular viability (Table 3.2), thus confirming that the observed changes in expression was not due to toxic effect of the inhibitor, but its specific action on the PI3K-dependent pathway.

(A)



(B)

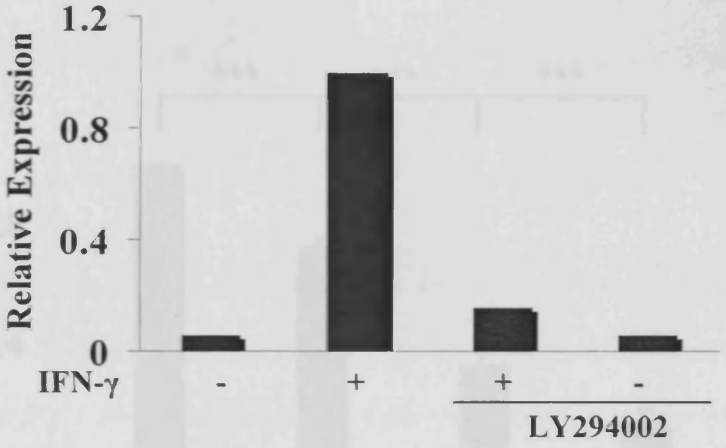


Figure 3.7 Effect of LY294002 on the regulation of MCP-1 mRNA expression by IFN-γ

PMA-differentiated THP-1 macrophages were pre-treated for 1h with LY294002 (20μM) or DMSO as a vehicle control. The cells were then cultured for 3h in the presence or the absence of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1 and GAPDH as shown. The amplification products were analysed by agarose gel electrophoresis and the images are representative of two independent experiments (A). M denotes the 1kb DNA ladder (Appendix I). Panel C shows the average relative expression of MCP-1 normalised to the expression GAPDH (IFN-γ-treated, DMSO control assigned as 1) from two independent experiments.

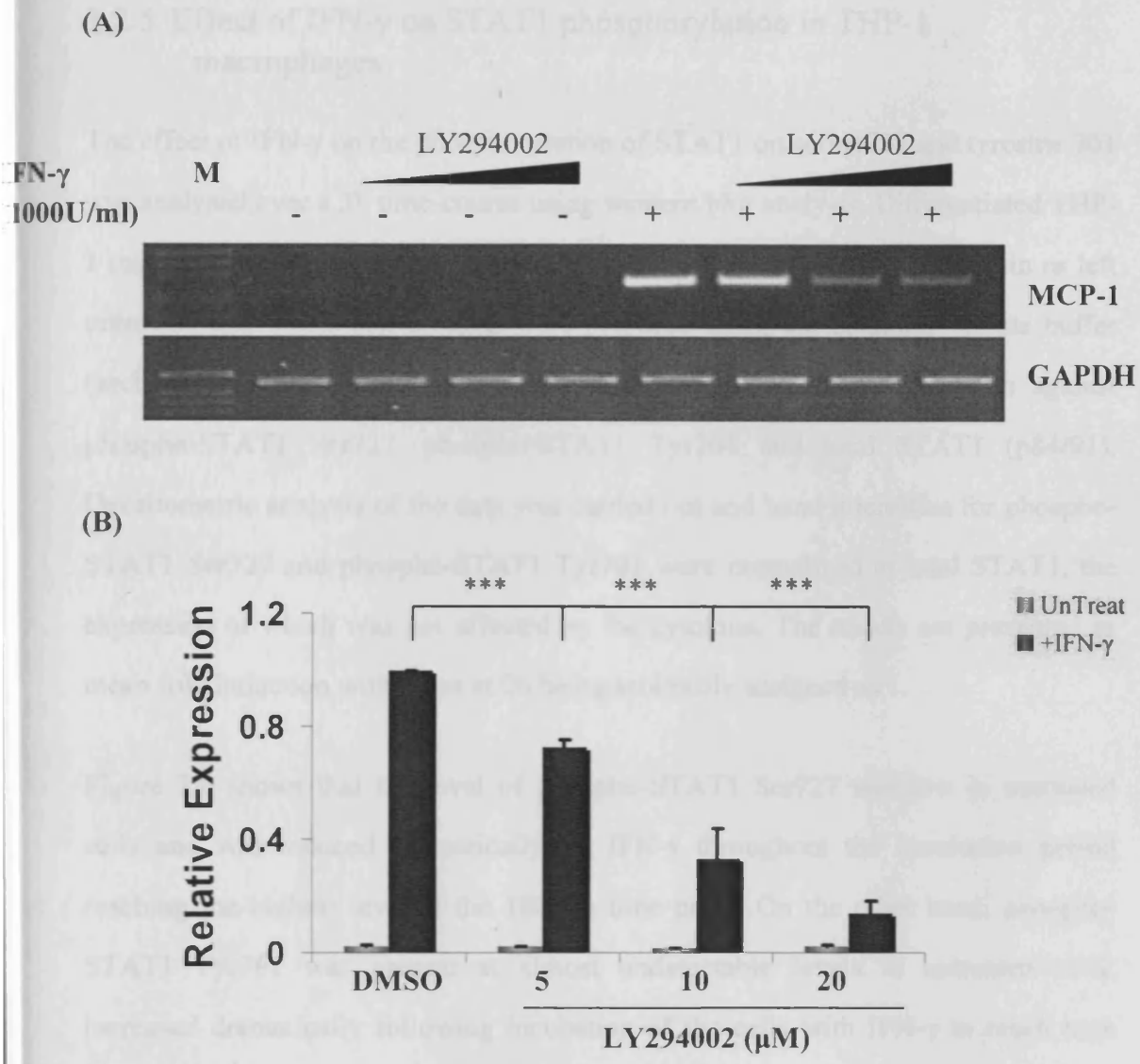


Figure 3.8 Concentration-dependent effect of LY294002 on the regulation of MCP-1 mRNA expression by IFN-γ

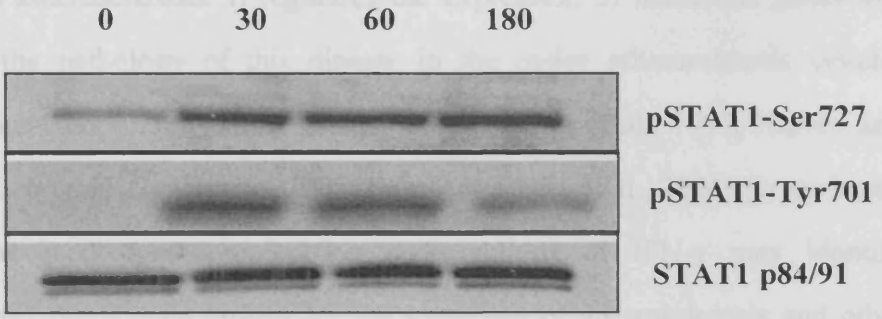
PMA-differentiated THP-1 macrophages were pre-treated for 1h with LY294002 (5, 10 and 20μM) or DMSO as a vehicle control. The cells were then cultured for 3h in the presence or the absence of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1 and GAPDH. The amplification products were analysed by agarose gel electrophoresis and the images shown are representative of three independent experiments (A). M denotes the 1kb DNA ladder (Appendix I). Panel B shows the relative expression (mean ±SD) of MCP-1 normalised to the expression of GAPDH (IFN-γ-treated, DMSO control assigned as 1) from three independent experiments. Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with LY294002 and IFN-γ (***, $P<0.001$).

3.2.5 Effect of IFN-γ on STAT1 phosphorylation in THP-1 macrophages

The effect of IFN-γ on the phosphorylation of STAT1 on serine727 and tyrosine 701 was analysed over a 3h time-course using western blot analysis. Differentiated THP-1 macrophages were incubated with IFN-γ for periods of 30, 60 and 180min or left untreated and whole cell proteins were prepared using the Laemmli sample buffer (section 2.6.2). Western blot analysis was carried out using antibodies against phospho-STAT1 Ser727, phospho-STAT1 Tyr701 and total STAT1 (p84/91). Densitometric analysis of the data was carried out and band intensities for phospho-STAT1 Ser727 and phospho-STAT1 Tyr701 were normalized to total STAT1, the expression of which was not affected by the cytokine. The results are presented as mean fold induction with those at 0h being arbitrarily assigned as 1.

Figure 3.9 shows that the level of phospho-STAT1 Ser727 was low in untreated cells and was induced dramatically by IFN-γ throughout the incubation period reaching the highest level at the 180min time point. On the other hand, phospho-STAT1 Tyr701 was present at almost undetectable levels in untreated cells, increased dramatically following incubation of the cells with IFN-γ to reach high levels at 30-60min and declined at 180min. The 30min period was selected for future studies as this was the earliest time point where high levels of both phospho-STAT1 Ser727 and phospho-STAT1 Tyr701 were observed.

(A)



(B)

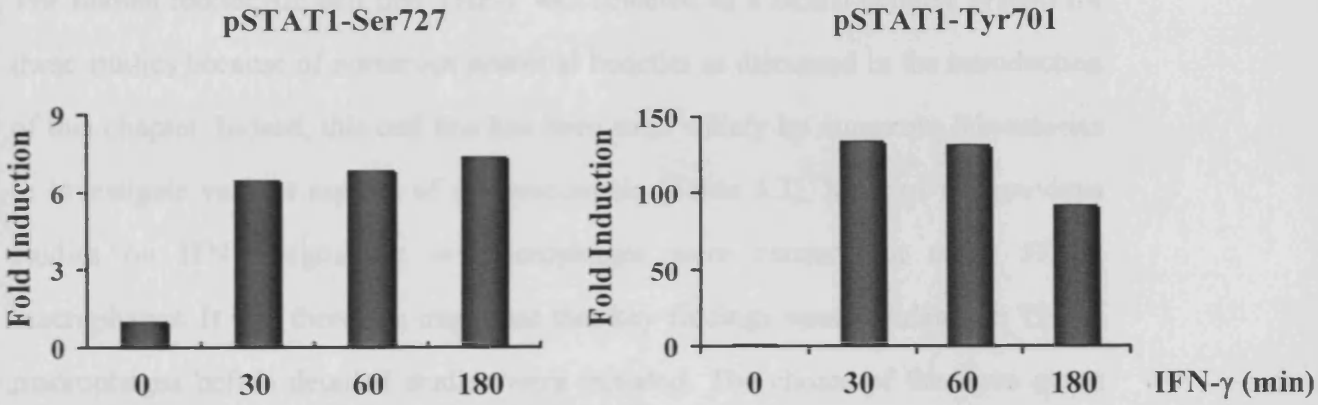


Figure 3.9 Effect of IFN-γ on the phosphorylation of STAT1 at serine 727 and tyrosine 701 over time

PMA-differentiated THP-1 macrophages were treated with IFN-γ for the indicated period of time and whole cell protein extracts were prepared. Western blot analysis was carried out using antibodies specific for phospho-STAT1 Tyr701, phospho-STAT1 Ser727 and STAT1 p84/91 (A). Panel B shows the average fold induction in the phosphorylation level of STAT1 at these sites (Ser727 and Tyr701) normalised to the expression of the total STAT1 protein level as determined by densitometric analysis from two independent experiments. The values for the 0h control have been arbitrarily assigned as 1.

3.3 Discussion

The inflammatory cytokine IFN-γ has fundamental and complex roles in the progression of atherosclerosis. It regulates the expression of numerous genes that contribute to the pathology of this disease in the major atherosclerotic cellular components, most importantly macrophages [see (Boehm et al., 1997; Harvey and Ramji, 2005; Leon and Zuckerman, 2005; Schroecksnadel et al., 2006) for reviews]. Studies of the mechanisms underlying such actions of IFN-γ may identify potentially novel therapeutic targets for the treatment of atherosclerosis and other inflammatory diseases.

The human monocytic cell line THP-1 was selected as a model cellular system for these studies because of numerous potential benefits as discussed in the introduction of this chapter. Indeed, this cell line has been used widely by numerous laboratories to investigate various aspects of atherosclerosis (Table 3.3). Most of our previous studies on IFN-γ signalling in macrophages were carried out using J774.2 macrophages. It was therefore important that key findings were validated in THP-1 macrophages before detailed studies were initiated. The choice of the three genes selected for the initial experiments (MCP-1, SOCS-1 and LPL) was based on their key roles in atherosclerosis and potential regulation by different mechanisms. For example, MCP-1 and SOCS-1 are both recognised as being regulated by IFN-γ through the action of STAT1 (Valente et al., 1998; Zhou et al., 1998; Zhou et al., 2001; Alexander, 2002; Ramana et al., 2002). However, Gil et al. (2001) have shown that MCP-1 expression is up-regulated by IFN-γ in both wild type and STAT1-deficient cells, thereby suggesting potential roles of STAT1-independent pathways, whereas SOCS-1 expression was not induced by IFN-γ in STAT1-null macrophages (Ramana et al., 2002). The inhibition of LPL expression in response to IFN-γ has been studied previously in our laboratory and has resulted in the identification of a potentially important role for CK2 and PI3K through the

modulation of binding of the transcription factors Sp1/Sp3 to regulatory regions of this gene (Hughes et al., 2002; Harris et al., 2008). CK2 and PI3Ks were also found to be involved in the IFN-γ-mediated induction of MCP-1 and SOCS-1 expression through the regulation of STAT1 phosphorylation at serine 727 (Harvey et al., 2007).

The action of IFN-γ on the expression of MCP-1, SOCS-1 and LPL in THP-1 macrophages was investigated initially by time course RT-PCR experiments. Consistent with previous studies in J774.2 macrophages, IFN-γ induced the expression of MCP-1 and SOCS-1, but reduced that for LPL (Figure 3.3). The kinetics for IFN-γ action on these genes was similar to that observed in mouse macrophages (Hughes et al., 2002; Harvey et al., 2007). The expression of MCP-1 and SOCS-1 peaked at 6h, and were reduced at subsequent time points, thereby suggesting the existence of further signalling events that inhibit MCP-1 and SOCS-1 expression. SOCS-1 itself is likely to be responsible for this inhibition through a negative feedback mechanism.

Macrophage LPL is recognised as a pro-atherogenic mediator and inhibition of its expression therefore represents an athero-protective role for IFN-γ. Although widely recognized as a pro-inflammatory cytokine, IFN-γ also possesses some anti-inflammatory and anti-atherogenic properties (Harvey and Ramji, 2005; McLaren and Ramji, 2009), such as inhibition of macrophage LPL expression and the oxidation of LDL (Christen et al., 1994; Fong et al., 1994). However, when all the evidence is taken into account, particularly studies emerging from mouse models (section 1.5 and Table 1.3), it is clear that the major action of IFN-γ is pro-atherogenic (Harvey and Ramji, 2005; McLaren and Ramji, 2009). It was therefore decided to focus on the pro-atherosclerotic aspects of IFN-γ for future studies. MCP-1, a key chemokine in this disease, was chosen here for further research with the use of pharmacological inhibitors.

Table 3.3 List of publications utilising THP-1 to study various aspects of atherosclerosis

Aspects of atherosclerosis	References
Adhesion of monocytes to endothelial cells	(Redmond et al., 2009); (Guo et al., 2009); (Chen et al., 2008); (Tikellis et al., 2008)
Cellular apoptosis	(Liu et al., 2007a); (Berthier et al., 2005); (Cappello et al., 2007); (Baird et al., 2005); (Geng et al., 2003); (Inagaki et al., 2002); (Liao et al., 2000)
Foam cell formation	(Kang et al., 2008); (Kang et al., 2006); (Hu et al., 2008); (Wang et al., 2009); (Osto et al., 2008); (Ning et al., 2009); (Crow et al., 2008); (Tian et al., 2009); (Wang et al., 2008); (Ohwaki et al., 2007)
Immune responses	(Dasu et al., 2008); (Ibeas et al., 2009); (Fuentes et al., 2002); Kim, et al. 2008; (Holvoet et al., 2006); (Figarola et al., 2007); (Bonta et al., 2006)
Monocyte migration	(Wagsater et al., 2009) (Chen and Cheng, 2007); (Guo et al., 2009); (Liu et al., 2008)
NR action	(Hao et al., 2009); (Inoue et al., 2001); (Ning et al., 2009) (Liu et al., 2007b); (Qiu and Hill, 2008); (Hu et al., 2006); (Sevov et al., 2006); (Seki et al., 2005)

It has previously been shown that following 24h incubation, IFN-γ can promote cell death of THP-1 macrophages under certain culture conditions (0.1% (v/v) FCS, 50μM β-mercaptoethanol and pre-treatment with 100 ng/ml PMA for 2 days) (Inagaki et al., 2002). This is clearly not the case in this study. According to the trypan blue exclusion assay, approximately 98% of the cells are viable following culturing in growth media with 10% FCS, differentiation by treatment with 160ng/ml PMA for 24h and incubation for 3h with IFN-γ (1000U/ml). In the light of this finding and maximal induction of MCP-1 expression (Figure 3.4), it was decided to use this concentration of IFN-γ for future studies.

Consistent with previous findings in the J774.2 cell line, pre-incubation of THP-1 macrophages with apigenin (inhibitor for CK2) and LY294002 (inhibitor for PI3K) dramatically attenuated the IFN-γ-induced up-regulation of MCP-1 gene expression in a concentration dependent manner. These results suggest that similar to mouse macrophages, CK2- and PI3K-dependent pathways play crucial roles in the IFN-γ-stimulated expression of the MCP-1 gene in THP-1 macrophages. The potential importance of these pathways in relation to MCP-1 expression have also been confirmed previously in human primary monocyte-derived macrophages (Harvey et al., 2007), indicating the direct relevance of these studies to conditions *in vivo* and ruling out any potential cell line-specific effects. As detailed above, MCP-1 gene expression has been found to be induced by IFN-γ in both wild type and STAT1-null cells, thereby suggesting the potential existence of STAT1-independent pathways regulating its expression (Gil et al., 2001). However, our previous studies showed that the action of CK2 and PI3K on MCP-1 expression was mediated, at least in part, through the regulation of STAT1 serine727 phosphorylation (Harvey et al., 2007). It is at present unclear whether CK2 and PI3K/Akt act directly on STAT1 or through other downstream effector kinases.

Despite the existence of numerous studies that have used LY294002 as a specific inhibitor for PI3K (Birkenkamp et al., 2000; Nguyen et al., 2001; Choudhury, 2004;

Harvey, 2006; Venkatesan et al., 2006; Lin et al., 2007), Choi et al. (2004) showed that LY294002 at a high concentration (50μM) could mediate inhibition of IL-1-stimulated MCP-1 expression independently of PI3K in phorbol ester-differentiated THP-1 macrophages. However, the specific action of LY294002 at concentrations used in this study (10-30μM) on PI3K-dependent pathway has previously been confirmed in our laboratory. It has been shown that the IFN-γ-induced activation of Akt was inhibited by LY294002 (Harvey, 2006). Moreover, the role of PI3K/Akt in the IFN-γ-induced MCP-1 expression has been confirmed via an alternative approach in our laboratory. It was shown that transfection of the cells with a DN form of Akt resulted in a large decrease in the IFN-γ-induced activation of a DNA construct containing the MCP-1 promoter linked to the luciferase reporter gene (MCP[213]Luc) as well as the IFN-γ-mediated activation of a plasmid with three tandem STAT1 consensus sites (GAS) upstream of a minimal promoter and the luciferase reporter gene (Harvey et al., 2007). In order to further confirm the role of the PI3K pathway in IFN-γ signalling in human macrophages, the effect of LY294002 on the IFN-γ-induced STAT1 phosphorylation was investigated in THP-1 cells and the results are presented in Chapter 4 (section 4.2.2).

Consistent with previous findings in a range of cell types (Goh et al., 1999; Overbergh et al., 2006; Kanda and Watanabe, 2007), IFN-γ elicited the phosphorylation of STAT1 at Ser727 and Tyr701 in THP-1 macrophages and phosphorylation at Ser727 occurred with a more delayed kinetics compared to that for tyrosine phosphorylation (Figure 3.9). Phosphorylation at the tyrosine residue is known to be obligatory for STAT dimerisaton (Shuai et al., 1994), nuclear translocation and subsequent DNA binding activity (Shuai et al., 1993), whereas the exact role of serine phosphorylation is relatively poorly understood despite the fact that it is essential for maximal transcriptional activity (Wen et al., 1995). Serine phosphorylation has been claimed not to contribute to DNA binding (Wen and Darnell, 1997), but might be essential for the recruitment of transcriptional co-

activator, such as CBP/p300 (Sun et al., 2005). The differential function of tyrosine and serine phosphorylation might explain the delayed kinetics of phosphorylation of serine compared to that of tyrosine. The maximal phosphorylation at both Tyr701 and Ser727 sites was observed at 30min and 180min respectively. This is consistent with an important role for this transcription factor in the regulation of MCP-1 and SOCS-1 expression, which is induced maximally after 6h of cytokine treatment (Figure 3.3).

In summary, the work presented in this chapter shows that we have successfully reproduced and extended the previous findings established in our laboratory using J774.2 macrophages to human THP-1 macrophages. First, IFN-γ has been shown to regulate the expression of MCP-1, SOCS-1 and LPL genes with similar kinetics to that found in mouse J774.2 macrophages. Also in support with previous findings, CK2- and PI3K-dependent pathways have been demonstrated to play important roles in the regulation of MCP-1 expression in human macrophages. Moreover, IFN-γ induced the phosphorylation of STAT1 at Ser727 and Tyr701 in THP-1 macrophages in a pattern similar to that observed in mouse macrophages and human primary monocyte-derived macrophages. These results thereby suggest that THP-1 macrophages provide a useful and validated cellular model system for further studies of IFN-γ signalling pathway.

STAT1 Ser727 phosphorylation is known to be required for its full transcriptional activity (Wen et al., 1995). Unfortunately, the mechanisms responsible for the regulation of phosphorylation at this site are poorly understood, particularly in macrophages. It is therefore of great interest to identify the exact kinases that act as upstream mediators of STAT1 activation and are responsible for serine phosphorylation in response to IFN-γ. This aspect was the focus of further studies in the next chapter.

CHAPTER FOUR:

ROLES OF ERK AND JNK IN IFN-γ SIGNALLING

Chapter 4: Roles of ERK and JNK in IFN-γ signalling

4.1 Introduction

STAT1 is one of the most extensively studied transcription factors that are involved in IFN-γ-stimulated signalling pathways. Activation of STAT1 is largely controlled by phosphorylation at Tyr701 and Ser727 (Krause et al., 2006). Our current understanding of the mechanisms by which IFN-γ mediates STAT1 phosphorylation at Ser727 is relatively poor, although several studies have suggested that a number of kinases, such as MAPK, PKC-δ and CaMK II are likely to be involved (Nair et al., 2002; Deb et al., 2003; Overbergh et al., 2006; Kanda and Watanabe, 2007) (see section 1.6.2.1). Our previous studies have identified critical roles for CK2 and PI3K in the IFN-γ-regulated expression of several genes in mouse macrophages, and the action of both kinases was mediated, at least in part, through the regulation of Ser727 phosphorylation on STAT1 (Mead et al., 2003; Harvey et al., 2007). However, contradictory results have also been documented in relation to kinases that phosphorylate STAT1 on Ser727. For example, Kovarik et al. (2001) showed that the phosphorylation on STAT1 at Ser727 occurred in IFN-γ-stimulated mouse fibroblasts without a need for p38 MAPK, ERK1/2 or JNK. In addition, Valledor (2008) demonstrated that ERK and JNK selectively regulate the IFN-γ-mediated gene expression in mouse BMMs independently of STAT1 Ser727 phosphorylation. It is thus possible that these kinases exert their role in IFN-γ signalling in a cell type-specific manner. Potential roles for MAPK, CK2 and PI3K in IFN-γ-mediated signalling have been detailed in sections 1.7 and 3.1.3-4, and those for PKC-δ and CaMK II are discussed below.

4.1.1 PKC-δ

PKC-δ belongs to a family of serine/threonine protein kinases (PKC) consisting of over 12 iso-enzymes. These are divided into three groups: conventional, novel and

atypical, based on their structure along with second messenger and co-factor requirements. PKC-δ is known as a novel PKC (nPKC), and involved in a diverse range of biological responses including cellular growth, apoptosis, immune responses and tissue remodeling [see (Dempsey et al., 2000; Harper and Poole, 2007; Reyland, 2007) for reviews]. A plethora of studies have proposed important roles for PKC-δ in processing of signals triggered by extracellular effectors [see (Gschwendt, 1999; Rybin et al., 2004) for reviews]. In particular, it has been suggested that PKC-δ acts as a downstream effector of the PI3K pathway in the regulation of IFN-γ-stimulated gene transcription, potentially by mediating STAT1 Ser727 phosphorylation (Deb et al., 2003). An important role for PKCs in the IFN-γ-regulated ICAM-1 expression has also been demonstrated in epithelial cells, although the specific isoform responsible for this was not conclusively identified (Chang et al., 2002). In addition to STAT1, PKC-δ has been proposed to activate a number of other proteins involved in gene transcription, including STAT3, NFκB and p300 (Steinberg, 2004), all of which have previously been reported to mediate IFN-γ signalling in certain cellular systems (Zhang et al., 1996; Deb et al., 2001; Park et al., 2004).

4.1.2 CaMK II

CaMK II is a ubiquitously expressed serine/threonine protein kinase that is central to Ca^{2+} signalling. The mammalian CaMK II family is encoded by four genes (α, β, γ and δ) with further diversity created by alternative splicing. (Griffith, 2004a). CaMK II has a unique holoenzyme structure and autoregulatory properties that allow it to produce a prolonged response to transient Ca^{2+} signals, even after its activator CaM, dissociates, and to sense cellular Ca^{2+} oscillations [see (Hudmon and Schulman, 2002; Griffith, 2004b) for reviews]. Mouse genetic approaches have provided insight into the key function of these kinases, mainly on hippocampal plasticity, cortical plasticity, learning and memory (Elgersma et al., 2004). CaMK II is known

to phosphorylate a wide range of substrates, among which are transcription factors such as C/EBPβ, CREB and ATF, thereby suggesting a broad role of the kinase in the regulation of gene expression (Soderling et al., 2001). Of note, a recent study by Nair et al. (2002) showed that CaMK II, along with Ca^{2+} flux, is required for STAT1 Ser727 phosphorylation and associated downstream gene transcription in response to IFN-γ.

4.1.3 Experimental strategy

Work in this chapter was carried out with the aim of determining the potential kinases that act upstream of STAT1 in IFN-γ signalling and their roles in the IFN-γ-regulated gene expression and cellular responses in human macrophages. To initiate these investigations, it was decided to make use of commercially available pharmacological inhibitors designed against various kinases potentially involved in IFN-γ signalling pathways and screen for their effects on the IFN-γ-mediated STAT1 phosphorylation. The kinase(s) whose inhibition resulted in the most pronounced suppression of STAT1 phosphorylation were selected for future studies. The roles of the chosen kinases were firstly confirmed using DN mutants in transfection experiments. Moreover, additional pharmacological inhibitors were employed wherever necessary. Furthermore, the activation of these kinases by IFN-γ and their inhibition by the pharmacological agents used was investigated. Finally, the studies were extended to the investigation of the role of these pathways in the IFN-γ-mediated changes in gene expression and cellular responses relevant to atherosclerosis (e.g. adhesion of monocytes to endothelial cells) (see Figure 4.1 for overall strategy). The inhibitors that were selected for use have been employed extensively in many such studies, in a variety of cellular systems. Table 4.1 lists some studies that have made use of these pharmacological inhibitors, particularly in relation to IFN-γ or other cytokine/stimuli-mediated gene/protein expression or

phosphorylation of proteins important in the regulation of gene expression (e.g. STATs).

4.1.4 Selection of genes for study

4.1.4.1 Chemokines

Following screening for kinases involved in the IFN-γ-stimulated STAT1 phosphorylation, their roles in the regulation of gene expression by this cytokine was investigated. These kinases could potentially regulate gene expression predominantly through the modulation of STAT1, or they may also represent novel components of STAT1-independent pathways in IFN-γ signalling. Therefore, to further study the exact roles of these kinases in IFN-γ signalling, a number of additional genes to MCP-1 and SOCS-1, discussed in the previous chapter (section 3.1.5), were analysed. Amongst these were the two chemokine genes, MIP-1β and IP-10. Crucial roles for these two chemokines in atherosclerosis have been detailed in General Introduction (section 1.4.2.4). Similar to MCP-1, the expression of both these chemokines has been shown to be up-regulated by IFN-γ in a variety of cell types (Luster and Ravetch, 1987; Gil et al., 2001; Ramana et al., 2001; Jabuulonska et al., 2002). Previous studies have, however, been controversial regarding the mechanisms through which IFN-γ regulates MIP-1β expression. Ramana et al. (2001) showed that the IFN-γ-dependent induction of MIP-1β occurs in wild-type mouse embryonic fibroblasts but not in STAT1-null cells, thereby suggesting that the expression of MIP-1β in response to IFN-γ requires the presence of STAT1. However, these authors observed different results with studies on mouse BMMs, where STAT1 was shown not to be obligatory for MIP-1β expression (Gil et al., 2001). These findings probably reflect cell-specific differences in the mechanism of action. In the case of IP-10, the IFN-γ-induced gene expression has been shown to be impeded in STAT1-null cells in both cellular systems, thereby suggesting a STAT1-dependent pathway in gene regulation (Gil et al., 2001; Ramana et al., 2001).

4.1.4.2 ICAM-1

In addition to chemokines, adhesion molecules are another group of proteins that largely regulate the recruitment of monocyte/macrophages into the arterial intima via transendothelial migration (Engelhardt and Wolburg, 2004). Intercellular adhesion molecule-1 (ICAM-1) is an important member of this family of proteins and plays crucial roles during the development of atherosclerosis (Blankenberg et al., 2003). The expression of this molecule is detectable in macrophages, endothelial cells and SMCs in human atherosclerotic plaques (Poston et al., 1992). ICAM-1 has been found to promote the firm adhesion of monocytes, neutrophils, and lymphocytes to endothelial cells, principally by binding to the surface membrane β_2 integrin molecule, lymphocyte function associated antigen-1 (LFA-1) and macrophage antigen-1 (MAC-1) [see (Engelhardt and Wolburg, 2004; Liu et al., 2004b) for reviews]. The expression of ICAM-1 is low in quiescent cells, but markedly induced by inflammatory stimuli, such as IL-1, TNF-α and IFN-γ (Dustin and Springer, 1988; Yang et al., 2005). JAK1/2 and STAT1 have been shown to be required for the IFN-γ-induced ICAM-1 expression in endothelial cells (Chang et al., 2002, 2004). In keeping with this, Chung et al. (2002) reported that the IFN-γ-mediated transcription of ICAM-1 requires DNA sequences containing two major regulatory elements: a GAS sequence and SP1 sequence. Interestingly, Zhou et al. (1998) previously demonstrated that the STAT1-associated GAS element in MCP-1 promoter is required for the IFN-γ-induced expression, while SP1-bound GC-rich element is dispensible for IFN-γ responsiveness but obligatory for constitutive expression. It will thus be interesting to know whether a similar mechanism operates in the IFN-γ-mediated activation of ICAM-1 promoter as that required for MCP-1.

4.1.4.3 ICER

The mechanisms underlying the inhibition of gene transcription by IFN-γ remain largely unclear despite the existence of a large number of the genes that are regulated in this manner. Many of these genes are implicated in atherosclerosis, such

as LPL as discussed in the previous chapter (section 3.1.5.3). ICER has long been thought to be responsible for suppressing the expression of several genes regulated by IFN-γ through cAMP response elements (CREs) (Saavedra et al., 2000). The LPL gene for example, the down-regulation of which in response to IFN-γ is synergistic with cAMP, has putative ICER binding sites in its promoter (Enerback and Gimble, 1993). ICER is one of the products of the cAMP-response element modulator protein (CREM) gene that is produced by internal transcriptional initiation and alternative splicing. It belongs to a family of transcription factors that contain the basic-leucine zipper (bZIP) domain and mediates transcriptional regulation through binding to CREs in the promoter of target genes. ICER lacks a transactivation domain and hence represses gene transcription either by heterodimerisation with other family members or by competing with them for DNA-binding. Four isoforms of ICER (ICER I, ICER Iγ, ICER II and ICER IIγ), produced by alternative splicing of a transcript from the use of an internal promoter, have been discovered and appear to be functionally indistinct (Molina et al., 1993; Shaywitz and Greenberg, 1999; De Cesare and Sassone-Corsi, 2000). Our group has previously found that IFN-γ produces a dramatic increase in ICER expression in the mouse macrophage J774.2 cell line, potentially through the phosphorylation of CREB [or other CRE binding proteins] by CK2 (Mead et al., 2003), and this probably represents a JAK/STAT1-independent pathway (Harvey, 2006). Exploring the alternative kinases involved in such gene regulation could potentially contribute to our knowledge on the mechanisms responsible for the IFN-γ-mediated inhibition of gene transcription.

Table 4.1 List of publications utilising pharmacological inhibitors to study signalling pathways regulating cytokine/stimulus-mediated gene/protein expression

Inhibitor	Signalling Pathway	Cytokine(s) or other stimulus	Gene(s) or protein(s) analysed	Reference
PD98059	MEK/ERK	IFN-γ	B-site APP cleaving enzyme 1	(Cho et al., 2007)
		IFN-γ	25-hydroxyvitamin D-1α-hydroxylase	(Overbergh et al., 2006)
		IFN-γ	Procollagen	(Ghosh et al., 2006)
		IFN-γ	Inducible cAMP early repressor	(Mead et al., 2003)
		IFN-γ	Growth hormone	(Gong et al., 2003)
		IFN-γ	CCAAT-enhancer-binding protein β	(Salmenperä et al., 2003)
		IFN-γ and IL-1β	Hepatocyte growth factor	(Takami et al., 2005)
		IFN-γ and leptin	Nitric oxide synthase type II	(Otero et al., 2007)
		IFN-γ and LPS	Nitric oxide synthase and IL-12	(Zhu et al., 2006)
		IFN-γ and LPS	Inducible NO synthase	(Huang et al., 2004)
		IFN-γ and TNF-α	α (2A)-adrenoceptors	(Cayla et al., 2008)
		IL-17F	Interferon-γ–inducible protein 10	(Kawaguchi et al., 2007)
		IL-1β	Growth hormone	(Gong et al., 2005)
		TNF-α	Inter-cellular adhesion molecule 1 and Vascular cell adhesion molecule 1	(Hosokawa et al., 2006)
		TNF-α	Monocyte chemoattractant protein-1 and inter-cellular adhesion molecule 1	(Ho et al., 2008)
		angiotensin II and IL-1β	Osteopontin	(Xie et al., 2004)
U0126	MEK/ERK	IFN-γ	Nitric oxide synthase	(Mir et al., 2008)
		IFN-γ	Interferon-γ–inducible protein 10	(Dhillon et al., 2008)

		IFN-γ	A disintegrin and metalloproteinase 33	(Ito et al., 2007)
		IFN-γ	Interleukin-17E receptor	(Lajoie-Kadoch et al., 2006)
		IFN-γ	Bicistronic HCV replicon system	(Huang et al., 2006)
		IFN-γ	CCAAT-enhancer-binding protein β	(Salmenperä et al., 2003)
		IFN-γ and PMA	Inducible nitric oxide synthase	(Han et al., 2003)
		IL-17F	Interferon-γ–inducible protein 10	(Kawaguchi et al., 2007)
		LPS	Indoleamine 2,3-dioxygenase	(Jung et al., 2007)
		LPS	Class II, major histocompatibility complex transactivator	(Yao et al., 2006)
		LPS	Interleukin-6	(Ajuwon et al., 2004)
		TNF-α	Vascular endothelial growth factor	(Kim et al., 2009)
		TNF-α	Matrix metalloproteinase-9	(Kim et al., 2008)
SP600125	SAPK/JNK	IFN-γ and IL-1β	Hepatocyte growth factor	(Takami et al., 2005)
		IFN-γ and LPS	Inducible nitric oxide synthase	(Pawate and Bhat, 2006)
		IFN-γ and LPS	Inducible nitric oxide synthase	(Shibata et al., 2006)
		LPS	Indoleamine 2,3-dioxygenase	(Jung et al., 2007)
		LPS	Class II, major histocompatibility complex transactivator	(Yao et al., 2006)
		LPS	Interleukin-12 p70	(Nakahara et al., 2004)
			Tumor necrosis factor-α	
		LPS	Interleukin-1β	(Zhang et al., 2008)
			Tumor necrosis factor-α	
			Interleukin-6	

		TNF-α	Intercellular adhesion molecule 1 Vascular cell adhesion molecule 1	(Hosokawa et al., 2006)
		TNF-α	Growth hormone-inducible gene	(Buzzelli et al., 2008)
		TNF-α	Presenilin, nicastrin	(Kuo et al., 2008)
		TNF-α and granulocyte-macrophage colony-stimulating factor	Mcl-1 and XIAP	(Kato et al., 2008)
		TNF-α	Vascular endothelial growth factor	(Kim et al., 2009)
		TNF-α	Matrix metalloproteinase-9	(Kim et al., 2008)
		TNF-α	Monocyte chemoattractant protein-1	(Ho et al., 2008)
		angiotensin II and IL-1β	Intercellular adhesion molecule 1 Osteopontin	(Xie et al., 2004)
SB203580	p38 MAPK	IFN-γ	Interferon-γ–inducible protein 10	(Dhillon et al., 2008)
		IFN-γ	CCAAT-enhancer-binding protein β	(Salmenperä et al., 2003)
		IFN-γ and IL-1β	Hepatocyte growth factor	(Takami et al., 2005)
		IFN-γ and leptin	Nitric oxide synthase type II	(Otero et al., 2007)
		IFN-γ and LPS	Inducible nitric oxide synthase	(Shibata et al., 2006)
		IFN-γ and LPS	Monocyte chemoattractant protein-1	(Yoshimura and Takahashi, 2007)
		IFN-γ and LPS	Interferon regulatory factor Nitric oxide Inducible nitric oxide synthase	(Koide et al., 2007)
		LPS	Indoleamine 2,3-dioxygenase	(Jung et al., 2007)

		LPS	Class II major histocompatibility complex transactivator	(Yao et al., 2006)
		LPS	Interleukin-12 p70	(Nakahara et al., 2004)
		TNF-α	Tumor necrosis factor -α Matrix metalloproteinase-9	(Kim et al., 2008)
		TNF-α	Monocyte chemoattractant protein-1 Intercellular adhesion molecule 1	(Ho et al., 2008)
apigenin	CK2	IFN-γ	Inducible cAMP early repressor	(Mead et al., 2003)
		IFN-γ	Monocyte chemoattractant protein-1	(Harvey et al., 2007)
		TNF-α	Manganese superoxide dismutase	(Farah et al., 2003)
		angiotensin II and IL-1β	Osteopontin	(Xie et al., 2004)
		calpain	IkappaBα	(Shen et al., 2001)
		TGF-β	Apolipoprotein E	(Singh and Ramji, 2006)
LY294002	PI3K	IFN-α	Interferon-α target genes	(Hjortsberg et al., 2007)
		IFN-γ	Monocyte chemoattractant protein-1	(Harvey et al., 2007)
		IFN-γ	Signal transducers and activators of transcription 3	(Fang et al., 2006)
		IFN-γ	Monocyte chemoattractant protein-1	(Venkatesan et al., 2006)
		IFN-γ	Inducible nitric oxide synthase	(Hwang et al., 2004)
		IFN-γ	Signal transducers and activators of transcription 1 phosphorylation	(Choudhury, 2004)
		IFN-γ	p70 S6 kinase	(Lekmine et al., 2004)

		IFN-γ and leptin	Nitric oxide synthase type II	(Otero et al., 2007)
		IFN-γ and LPL	Signal transducers and activators of transcription-1	(Kota et al., 2005)
		IFN-γ and LPS	Inducible nitric oxide synthase, cyclooxygenase-2	(Sheu et al., 2005)
		IFN-γ and LPS	Inducible nitric oxide synthase	(Hattori et al., 2003)
		IFN-γ and LPS	Fas/Apo-1 and SP-100	(Kristof et al., 2003)
		IL-1β	Growth hormone gene	(Gong et al., 2005)
		LPS	Indoleamine 2,3-dioxygenase	(Jung et al., 2007)
Rottlerin	PKCδ	IFN-γ	Class II major histocompatibility complex transactivator	(Kwon et al., 2007)
		IFN-γ	Signal transducers and activators of transcription-1	(Deb et al., 2003)
		IFN-γ and LPS	Nitric Oxide	(Springael et al., 2007)
		IFN-α	Phospholipid scramblase 1	(Zhao et al., 2005)
		IFN-α	Leukemic CFU-GM progenitors	(Kaur et al., 2005)
		IFN-α and IFN-β	ISRE or GAS elements-controlled genes.	(Uddin et al., 2002)
		TNF-α	Intercellular adhesion molecule 1 Vascular cell adhesion molecule 1	(Woo et al., 2005)
		TNF-α and IL-1β	Nephrin	(Huwiler et al., 2003)
		TGFβ	Multipass transmembrane protein transporter ANK	(Cailotto et al., 2007)
		TGFβ	Fibronectin	(Ryer et al., 2006)
		TGFβ	Collagen I	(Runyan et al., 2003)
		IL-13	15-lipoxygenase	(Xu et al., 2004)

		IL-6	Signal transducers and activators of transcription 3 phosphorylation	(Schuringa et al., 2001)
KN93	CaMK II	IFN-γ	Signal transducers and activators of transcription 1 phosphorylation	(Nair et al., 2002)
		β-adrenergic receptor	Ca^{2+} release	(Oestreich et al., 2009)
		cytosolic calcium	p38 and activating transcription factor-2	(Wright et al., 2007)
		G protein-coupled receptor	Focal adhesion kinase	(Fan et al., 2005)
		hydrogen peroxide	Endothelial NO synthase Endothelial nitric oxide synthase	(Cai et al., 2004)
		hydrogen peroxide	Extracellular signal-regulated kinase 1/2 p38 MAPK Heat shock protein 27	(Nguyen et al., 2004)
		IL-1β	c-Jun	(Størling et al., 2005)
		light	Period gene	(Yokota et al., 2001)
		phorbol ester	IκB	(Hughes et al., 2001)

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; LPS, lipopolysaccharide MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinase; CK2, casein kinase 2; CaM K, Ca^{2+}/calmodulin-dependent protein kinase; PI3K, phosphoinositide 3-kinase

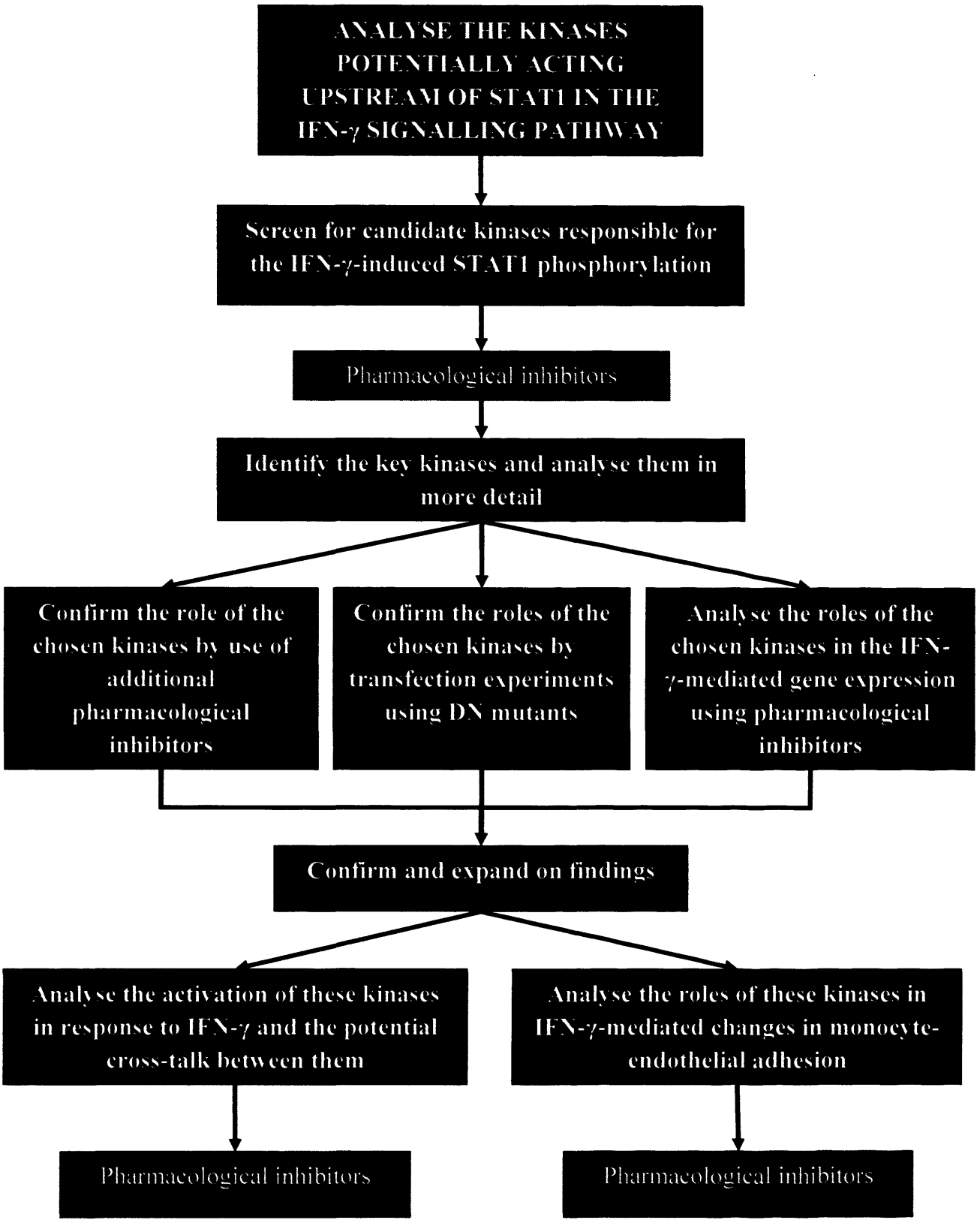


Figure 4.1 Experimental strategy

The aim of the experimental strategy was to analyse kinases acting upstream of STAT1 in the IFN-γ-mediated signalling pathway. This was achieved with the use of pharmacological inhibitors and DN mutants combined with analyses of alterations in STAT1 phosphorylation, promoter activation, mRNA expression, kinase activity and monocyte-endothelial cell adhesion.

4.2 Roles of the different kinases in the IFN-γ-induced STAT1 phosphorylation in macrophages

To identify kinases that are potentially involved in STAT1 phosphorylation, a range of pharmacological inhibitors for candidate kinases at a series of concentrations were employed. PMA-differentiated THP-1 macrophages were pre-treated with individual inhibitors for 1h prior to treatment with IFN-γ (1000U/ml) for 30min. The volume of DMSO added to the cells as a vehicle control was the same as that for the highest concentration of each inhibitor used in order to rule out the possibility of the effect of DMSO on STAT1 phosphorylation. Following incubation with the cytokine, whole cell proteins were extracted using the Laemmli sample buffer (section 2.6.2) followed by western blot analysis. Equivalent amount of whole cell proteins was subsequently subjected to western blot analysis with specific antibodies raised against phospho-STAT1 Ser727, phospho-STAT1 Tyr701 and STAT1 p84/91. Densitometric analysis of the data was carried out and presented in Figures 4.2-4.4. Band intensity for phospho-STAT1 Ser727 and Tyr701 were normalised to total STAT1 protein (p84/91). Results are presented as the IFN-γ-induced expression levels in the presence of inhibitors (±SD) normalized to induction level stimulated by the cytokine (arbitrarily assigned as 1). Where experiments were carried out three times or more, a Student's *t*-test was used to assess for statistical significance.

In experiments involving the use of inhibitors in THP-1 macrophages, the viability of the cells was analysed by the trypan blue exclusion assay. All the results showed a negligible proportion of dead cells (2-15%) following treatment with IFN-γ in combination with each of the inhibitor (Table 4.2), thereby indicating that the changes in protein or mRNA expression, if any, were not due to the cell death, but because of the specific action of the inhibitor.

	DM	DM	PD	U0126	SP	SB	rott	KN
IFN-γ	-	+	+	+	+	+	+	+
THP-1 viability (%)	100	100	98	98	95	97	90	96

Table 4.2 Assessment of viability of THP-1 macrophages following treatments with inhibitors

PMA-differentiated THP-1 cells were pre-treated for 1h with PD98059 (PD at 50μM), U0126 (20 μM), SP600125 (SP at 30μM), SB203580 (30μM), rottlerin (rott, 10μM) or KN93 (20μM) or DMSO (DM) as negative vehicle control. The cells were then either left untreated or exposed to IFN-γ (1000U/ml) for 30min. The viability of the cells was than assessed using the trypan blue exclusion assay. The data is obtained from a single experiment.

4.2.1 Effect of pharmacological inhibitors of MAPK on the IFN-γ-induced STAT1 phosphorylation

Figure 4.2-A and -B shows that pre-treatment of the cells with PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor) attenuated the IFN-γ-induced STAT1 phosphorylation at Ser727 in a concentration-dependent manner. At the highest concentration of PD98059 and SP600125 used (50μM and 30μM respectively), the level of phospho-STAT1 Ser727 was almost reduced to that observed in untreated cells. In contrast, the inhibitors had no effect on the IFN-γ-induced phosphorylation of STAT1 on Tyr701 in DMSO-treated control cells, thereby suggesting that the inhibitor did not affect the general phosphorylation status of proteins in the cells in a non-specific manner. Overall, the results suggest that ERK and JNK play important roles in the IFN-γ-mediated phosphorylation of STAT1 at Ser727, but not on Tyr701, in human macrophages.

In contrast to the ERK and JNK inhibitors, SB203580, a p38 MAPK inhibitor, was found not to impair either Ser727 or Tyr701 phosphorylation of STAT1 stimulated by IFN-γ (Figure 4.2C), thereby indicating that STAT1 activation by IFN-γ in human macrophages is potentially independent of p38 MAPK.

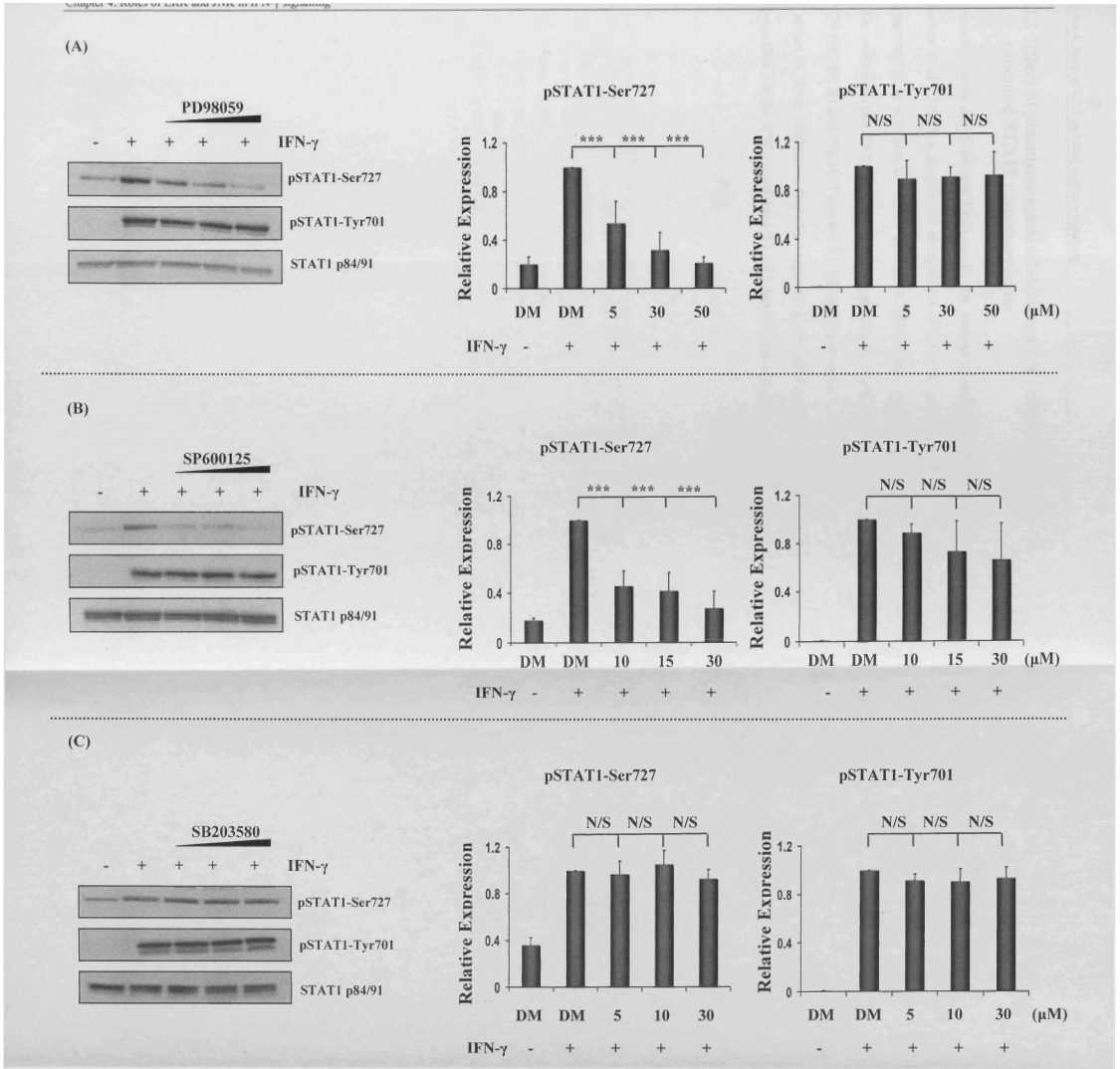


Figure 4.2 Effect of PD98059, SP600125 and SB203580 on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 macrophages were pre-treated with PD98059 (A), SP600125 (B) or SB203580 (C) at three different concentrations as indicated or DMSO (DM) as a vehicle control (volume corresponding to the highest concentration of the respective inhibitor used). They were then cultured in the presence or the absence of IFN-γ for 30min. Whole cell protein extracts were prepared and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701; pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1). Asterisks indicate significant attenuation of the IFN-γ-induced STAT1 phosphorylation in cells incubated with the DMSO control (***P<0.001; N/S, not significant)

4.2.2 Effect of pharmacological inhibitors of CK2 and PI3K on the IFN-γ-induced STAT1 phosphorylation

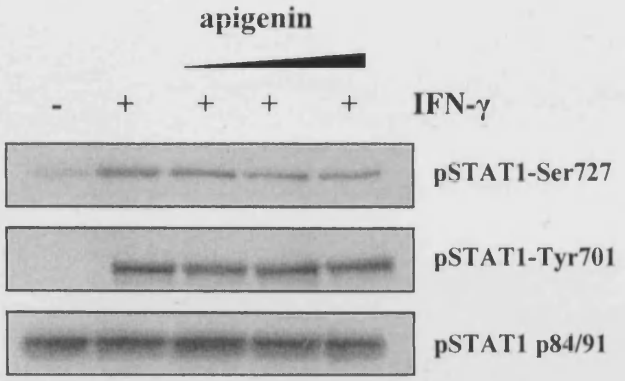
Consistent with our previous findings in the mouse macrophage J774.2 cell line (Harvey et al., 2007), a significant attenuation of the IFN-γ-induced STAT1 Ser727 phosphorylation was also observed in human THP-1 macrophages by pre-treatment of the cells with the CK2 inhibitor apigenin at 10 and 20μM, and the PI3K inhibitor LY294002 at 20 and 30μM (Figure 4.3). Phosphorylation at Tyr701 was not affected by either of these inhibitors, thereby suggesting that CK2 and PI3K are specifically responsible for the IFN-γ-mediated STAT1 Ser727 phosphorylation.

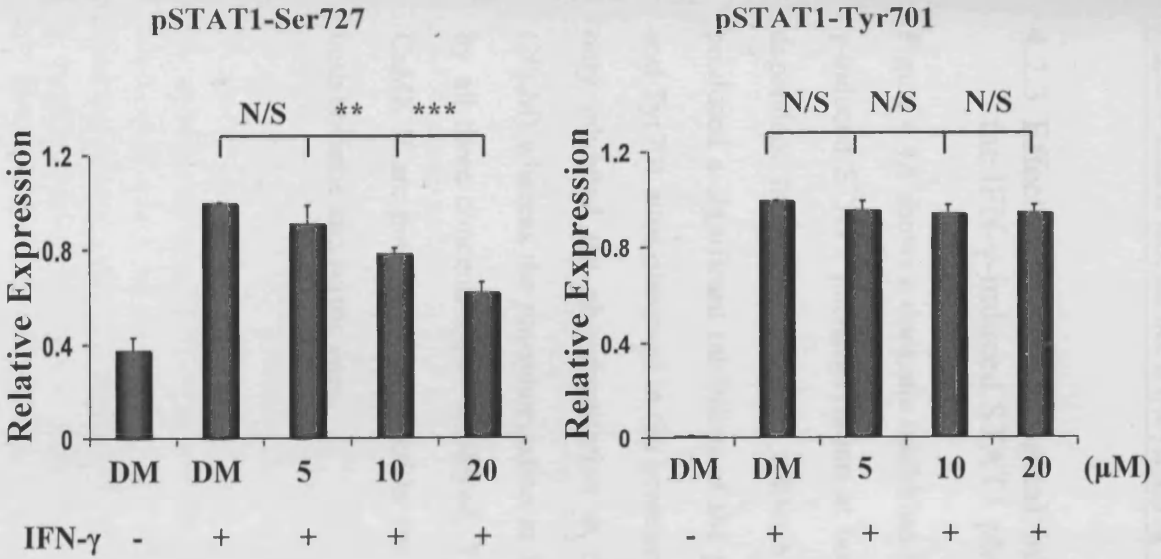
Figure 4.3 Effect of apigenin and LY294002 on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 macrophages were pre-treated with apigenin (A) and LY294002 (B) at three different concentrations as indicated or the DMSO control (volume corresponding to the highest concentration of the inhibitor was used) as a vehicle control. They were then cultured for 30min in the presence or the absence of IFN-γ. Whole cell protein extracts were prepared and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of total STAT1, as determined by densitometric analysis from three independent experimental series (value from IFN-γ-treated DMSO control cells has been arbitrarily assigned as 1). Asterisks indicate significant attenuation of the IFN-γ-induced STAT1 phosphorylation in control cells (**P<0.01; ***P<0.001; N/S, not significant)

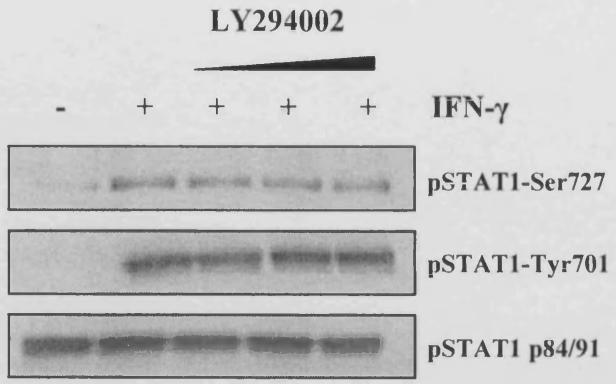
Chapter 4: Roles of ERK and JNK in IFN-γ signalling

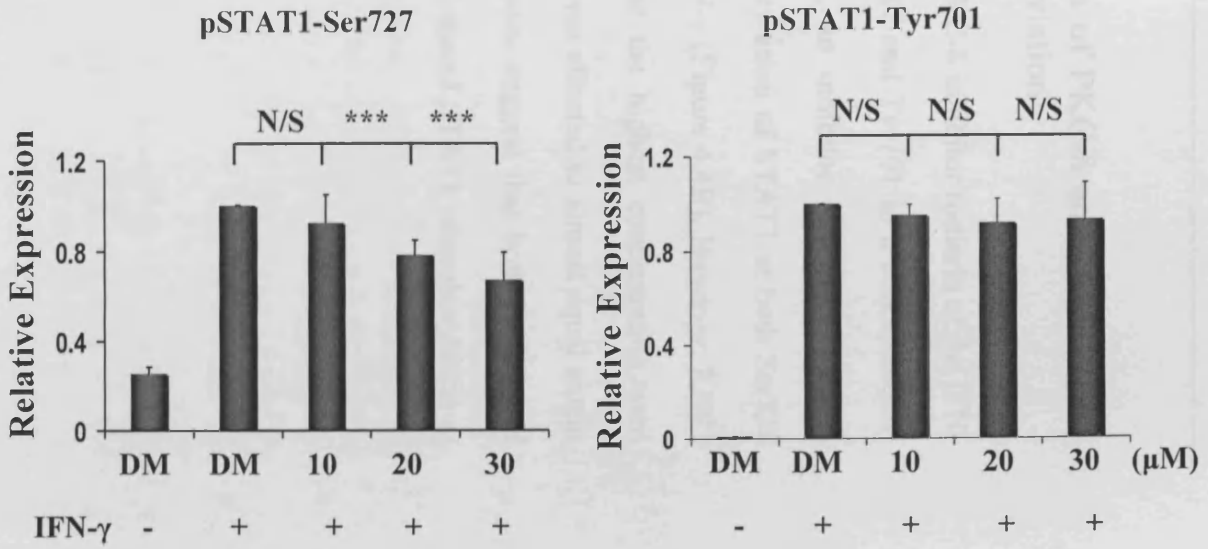
(A)





(B)



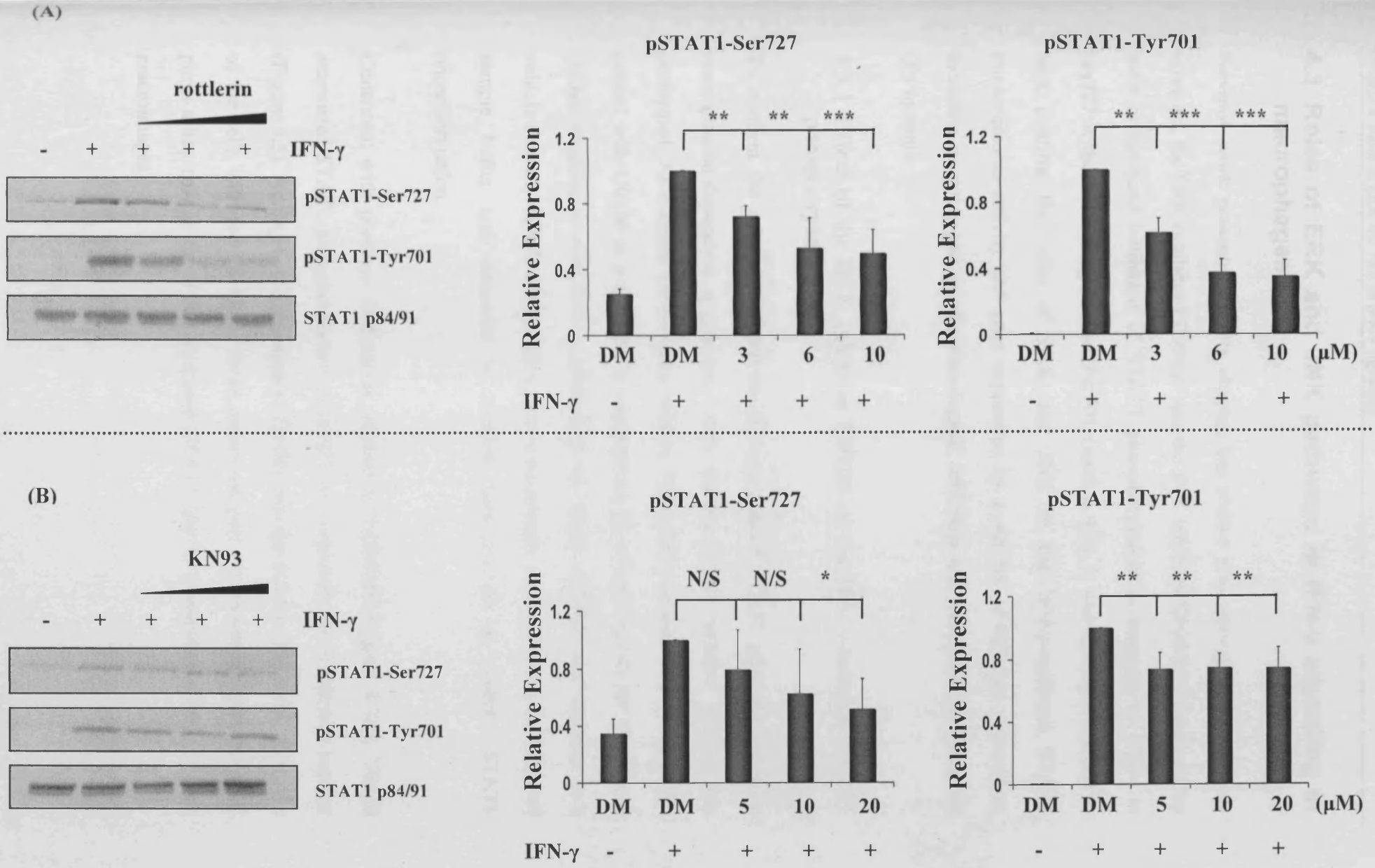


4.2.3 Effect of pharmacological inhibitors of PKC-δ and CaMK II on the IFN-γ-induced STAT1 phosphorylation

Figure 4.4A shows a dramatic inhibition by the PKC-δ inhibitor rottlerin of the IFN-γ-induced STAT1 phosphorylation at both Ser727 and Tyr701 in a concentration-dependent manner. Similar to rottlerin, KN93, an inhibitor of CaMK II, also produced a significant inhibition of the phosphorylation of STAT1 at both Ser727 and Tyr701 sites observed in the presence of IFN-γ (Figure 4.4B). However, KN93 only inhibited the phosphorylation at Ser727 at the highest concentration used (20μM) whereas the phosphorylation at Tyr701 was affected to almost equal extent by all three concentrations employed. These results suggest that both PKC-δ and CaMK II are potentially involved in the IFN-γ-induced STAT1 phosphorylation at both tyrosine and serine sites.

Figure 4.4 Effect of rottlerin and KN93 on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 macrophages were pre-treated with rottlerin (A) and KN93 (B) at three different concentrations as indicated or the DMSO control (volume corresponding to the highest concentration of the inhibitor was used). They were then cultured for 30min in the presence or the absence of IFN-γ. Whole cell protein extracts were prepared and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of total STAT1 as determined by densitometric analysis from three independent experimental series (the value from IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Asterisks indicate significant attenuation by the inhibitors of the IFN-γ-induced STAT1 phosphorylation observed in control cells (*P<0.05; **P<0.01; ***P<0.001; N/S, not significant).


(A)
rottlerin
- + + + + IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
pSTAT1-Tyr701
Relative Expression
1.2
0.8
0.4
0
** ** ***
** *** ***
DM DM 3 6 10
DM DM 3 6 10 (μM)
IFN-γ - + + + +
(B)
KN93
- + + + + IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
pSTAT1-Tyr701
Relative Expression
N/S N/S *
** ** **
DM DM 5 10 20
DM DM 5 10 20 (μM)
IFN-γ - + + + +

4.3 Roles of ERK and JNK pathways in IFN-γ signalling in macrophages

Previous work presented in this chapter has shown that amongst the inhibitors screened, the ERK inhibitor PD98059 and the JNK inhibitor SP600125 produced the most pronounced inhibition of STAT1 phosphorylation in response to IFN-γ at Ser727 without affecting that on Tyr701 (section 4.2). It was therefore decided to next confirm the roles of ERK and JNK in the IFN-γ-mediated STAT1 transcriptional activity and gene expression by a number of different approaches, including on-going use of pharmacological inhibitors and plasmids specifying for DN mutants.

4.3.1 Effect of the ERK inhibitor U0126 on the IFN-γ-induced STAT1 phosphorylation

To confirm the role of ERK in the IFN-γ-regulated STAT1 phosphorylation, a concentration-dependent experiment with another ERK inhibitor U0126 was performed. As detailed for previous studies, PMA-differentiated THP-1 cells were treated with U0126 at a series of concentrations (5, 10 and 20μM) for 1h prior to further stimulation with IFN-γ (1000U/ml) for 30min (DMSO was included as a vehicle control). Whole cell proteins were subsequently prepared using the Laemmli sample buffer and subjected to western blot analysis to monitor STAT1 phosphorylation.

Consistent with previous findings in relation to PD98059 (Figure 4.2A), U0126 attenuated STAT1 phosphorylation at Ser727 in a concentration-dependent manner (Figure 4.5). Again phosphorylation at Tyr701 was not affected following treatment of the cells with the inhibitor. These results support our hypothesis that the ERK plays a key role in the IFN-γ-mediated STAT1 Ser727 phosphorylation in human macrophages.

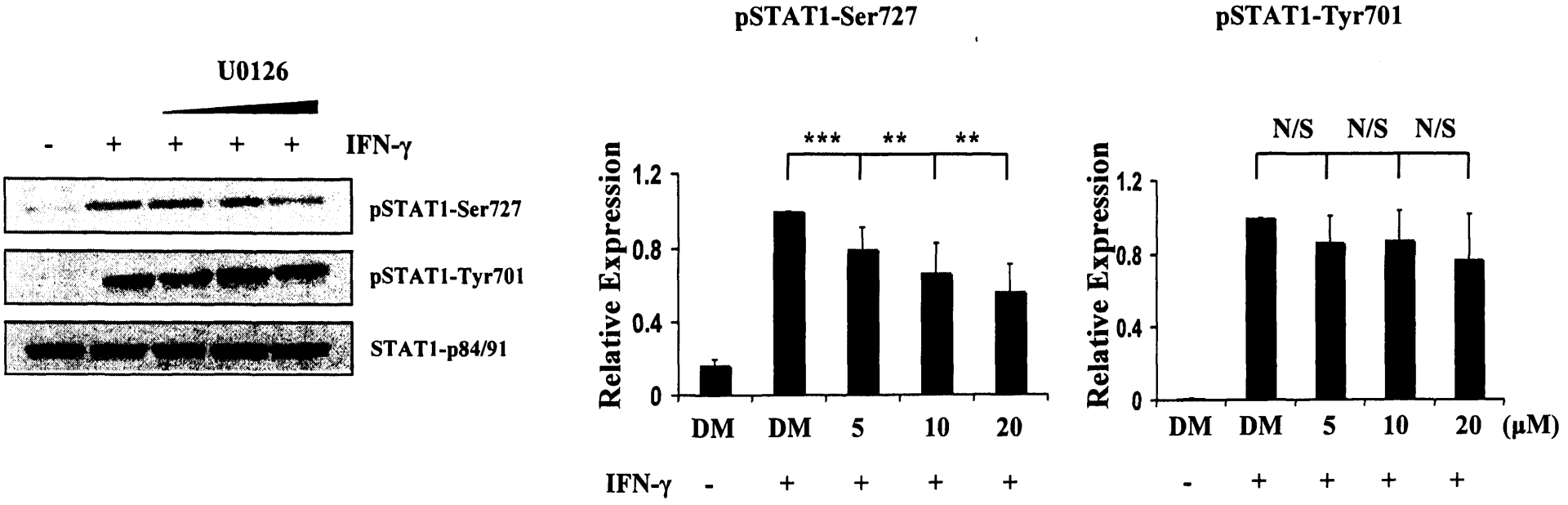


Figure 4.5 Effect of U0126 on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 macrophages were pre-treated with U0126 (5, 10 and 20μM) or DMSO (volume equivalent to 20μM of U0126) as a vehicle control. They were then cultured for 30min in the presence or the absence of IFN-γ. Whole cell protein extracts were prepared and western blot analysis was carried out using antibodies specific for: pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91 (A). The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at either of these sites normalised to the expression of total STAT1 as determined by densitometric analysis from three independent experimental series (the value from IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Asterisks indicate significant attenuation by the inhibitors of the IFN-γ-induced STAT1 phosphorylation observed in control cells (**P<0.01; ***P<0.001; N/S, not significant).

4.3.2 Further investigation into the role of ERK and JNK on the IFN-γ-mediated STAT1 phosphorylation in human primary monocyte-derived macrophages

Because the experiments so far were carried out on THP-1 macrophages, the action of IFN-γ and the inhibitors PD98059 and SP600125 on STAT1 phosphorylation in human primary monocyte-derived macrophages were investigated.

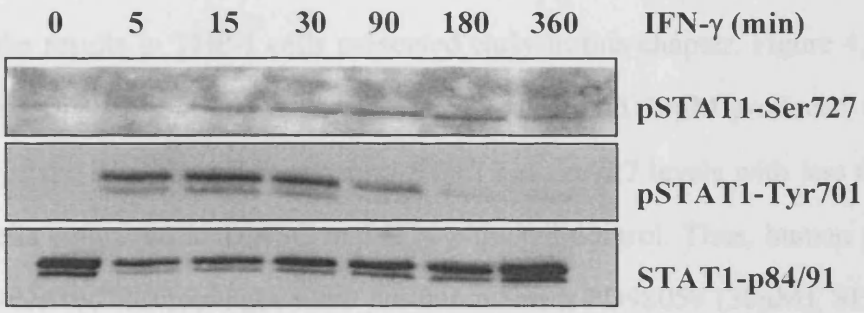
4.3.2.1 IFN-γ induced STAT1 phosphorylation in human primary monocyte-derived macrophages

Primary monocytes were isolated from buffy coats of human blood and allowed to differentiate in culture over seven days (section 2.3.11). The effect of IFN-γ on STAT1 Ser727 and Tyr701 phosphorylation was analysed over a 6h time-course using western blot analysis. Following differentiation, primary macrophages were incubated with IFN-γ for periods of 0, 5, 15, 30, 60, 90, 180 and 360min, after which whole cell protein was extracted using the Laemmli sample buffer (section 2.6.2). Western blot and densitometric analysis was carried out as detailed before and the results are shown in Figure 4.6.

Human primary macrophages showed the presence of low levels of phospho-STAT1 Ser727 and almost complete absence of phospho-STAT1 Tyr701. The level of phospho-STAT1 Tyr701 was induced dramatically by IFN-γ and reached the highest level at 5min following cytokine treatment. This was followed by reduction at subsequent time points to reach almost undetectable levels after 180min. The phosphorylation at Ser727 of STAT1 occurred with a delayed kinetics compared to Tyr701, but was nevertheless induced in a time-dependent manner. After reaching the highest level at 90min, there was a gradual decrease in a time-dependent manner. These results show that similar to THP-1 macrophages (Figure 3.9), IFN-γ induces the phosphorylation of STAT1 at both Tyr701 and Ser727. The 30min time point, which was chosen for further analysis of STAT1 phosphorylation in THP-1 cells, was again selected here for future work on human monocyte-derived macrophages.

Although there were some differences in total STAT1 levels between various time points, this is likely to be specific to this single experiment as previous studies consistently showed that IFN-γ does not affect the expression of total STAT1 in a vast range of cells, including human macrophages (Kovarik et al., 1998; Nguyen et al., 2000; Nguyen et al., 2001; Ramsauer et al., 2002; Overbergh et al., 2006; Harvey et al., 2007). As only a single experiment was performed, the variation in total STAT1 level could represent a technical error during the cellular protein extraction. In support to this, our subsequent analysis of the IFN-γ-induced STAT1 phosphorylation in human primary monocyte-derived macrophages showed that the total STAT1 protein levels did not change in response to this cytokine (Figure 4.7).

(A)



(B)

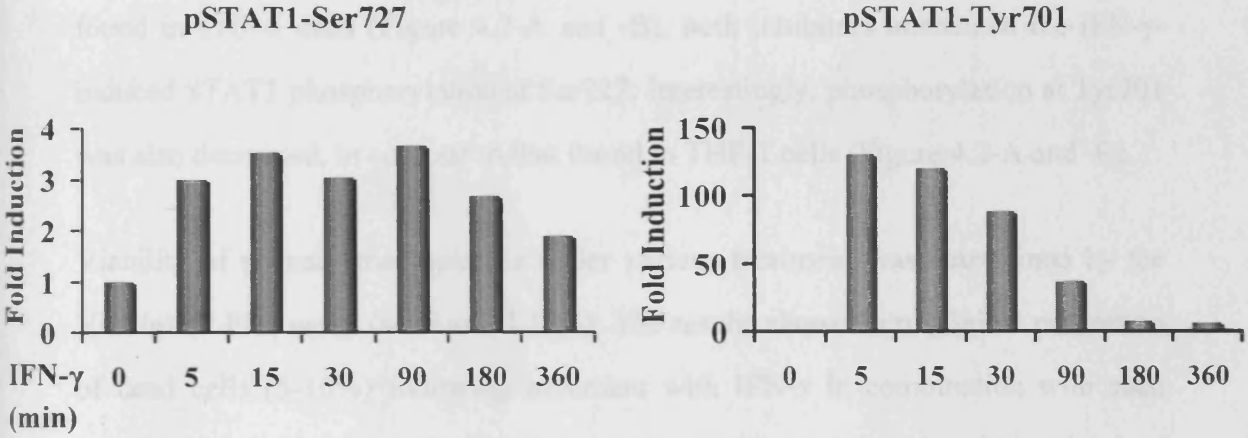


Figure 4.6 Time-course effect of IFN-γ on the phosphorylation of STAT1 at Ser727 and Tyr701 in human monocyte-derived macrophages

Human monocyte-derived macrophages were treated with IFN-γ for the indicated period of time and cellular protein extracts were prepared using the Laemmli sample buffer. Western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91 (A). Panel B shows the fold induction in the phosphorylation level of STAT1 at each of these sites normalised to the expression of total STAT1 protein levels, as determined by densitometric analysis (0min control assigned as 1) from this single experiment.

4.3.2.2 Effect of PD98059 and SP600125 on the IFN-γ-induced STAT1 phosphorylation in human primary monocyte-derived macrophages

The choice of concentration of inhibitors used here, PD98059 and SP600125, was based on the results in THP-1 cells presented early in this chapter. Figure 4.2A and B show that PD98059 at 30μM and SP600125 at 15 and 30μM produced 60-70% inhibition of the IFN-γ-induced phospho-STAT1 at Ser727 levels with less than 5% of dead cells compared to DMSO and IFN-γ-treated control. Thus, human primary monocyte-derived macrophages were pre-treated with PD98059 (30μM), SP600125 (20μM) or DMSO as a vehicle control. The cells were then cultured in the presence or the absence of human IFN-γ for 30min. Whole cell protein was extracted and western blot analysis was carried out as detailed before. Figure 4.7 shows that as found in THP-1 cells (Figure 4.2-A and -B), both inhibitors attenuated the IFN-γ-induced STAT1 phosphorylation at Ser727. Interestingly, phosphorylation at Tyr701 was also decreased, in contrast to that found in THP-1 cells (Figure 4.2-A and -B).

Viability of primary macrophages under various treatment was determined by the ViaLight® Plus assay (section 2.3.12.2). The results showed a negligible proportion of dead cells (5-10%) following treatment with IFN-γ in combination with each inhibitor (Table 4.3), suggesting that the changes in the phosphorylation level of STAT1 at both sites was not due to a toxic effect of the cytokine or the inhibitor.

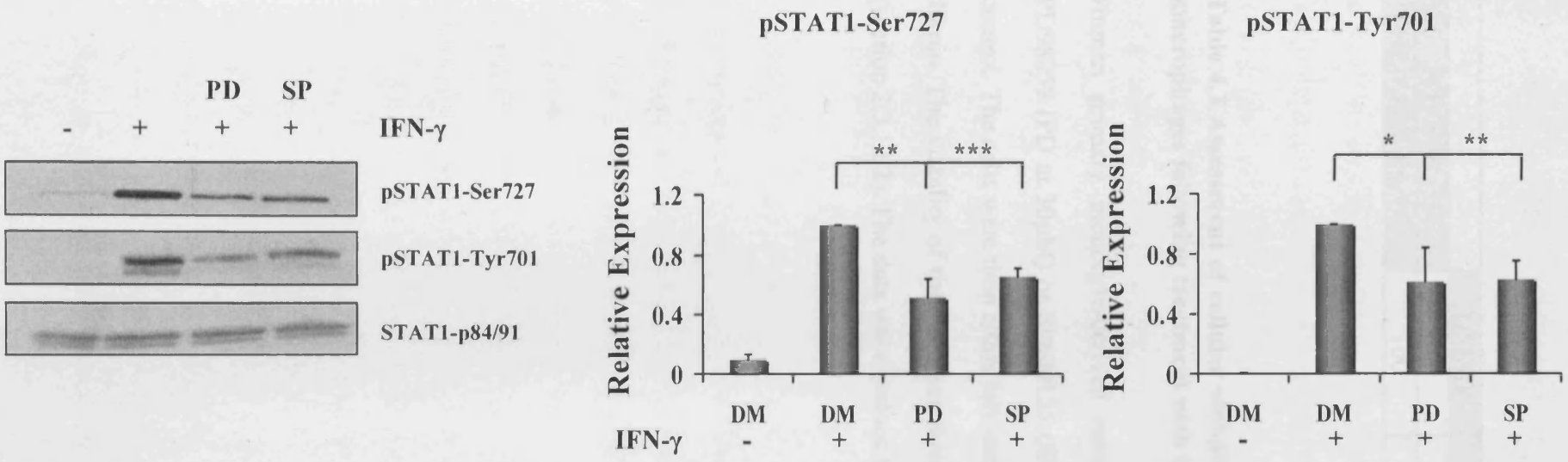


Figure 4.7 Effect of PD98059 (PD) and SP600125 (SP) on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701 in human monocyte-derived macrophages

Human monocyte-derived macrophages were pre-treated with PD98059 (30μM) and SP600125 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or the absence of IFN-γ for 30min. Cellular protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91 (A). The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at either of these sites normalised to the expression of total STAT1 as determined by densitometric analysis from three independent experimental series (the value from IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Asterisks indicate significant attenuation by the inhibitors of the IFN-γ-induced STAT1 phosphorylation observed in control cells (*P<0.05; **P<0.01; ***P<0.001).

	DMSO	DMSO	PD	SP
IFN-γ	-	+	+	+
THP-1 viability (%)	100	100	89	91

Table 4.3 Assessment of cellular viability of human primary monocyte-derived macrophages following treatment with inhibitors and IFN-γ

Human primary monocyte-derived macrophages were pre-treated for 1h with PD98059 (PD at 30μM) or SP600125 (SP at 20μM) or DMSO as negative vehicle control. The cells were then either left untreated or exposed to IFN-γ (1000U/ml) for 30min. The viability of the cells was than assessed using the ViaLight® Plus assay (section 2.3.12.2). The data was obtained from a single experiment.

4.3.3 Validation of a hepatocyte transfection system for studies on STAT1 transcriptional activity

Several studies have shown that macrophage cell lines are relatively difficult to transfect at high efficiency with exogenous DNA (Ohtani et al., 1989; Kusumawati et al., 1999; Dokka et al., 2000). Therefore, transfection based studies were carried out in the human hepatoma Hep3B cell line (section 2.3.3.2) using the SuperFect™ method (section 2.5.2). Experiments in our laboratory have demonstrated that this cell line can be transfected at high efficiency with exogenous DNA. Our research group has also shown that Hep3B cells provide a good model for the cytokine-regulated gene expression and responses to IFN-γ (Foka et al., 2003; Irvine et al., 2005; Ali, 2007; Harvey et al., 2007). In addition, Harvey (2006) has demonstrated that the level of induction of MCP-1 expression in Hep3B cells by IFN-γ and the inhibition of this response by LY294002 and apigenin was comparable to that seen in macrophages.

In order to further validate the use of this system in the study of STAT1 transcriptional activity in response to IFN-γ, western blot analysis was carried out to demonstrate the induction of STAT1 phosphorylation by IFN-γ and the effect of PD98059 on this. Hep3B cells were incubated with IFN-γ (100U/ml) for periods of 30, 60 and 180min or left untreated (the optimal concentration of the cytokine used in Hep3B cells has been previously determined by our group as 100U/ml (Harvey, 2006)), after which whole cell protein was extracted using the Laemmli sample buffer (section 2.6.2). Western blotting and analysis of the data was carried out as described before and the results are shown in Figure 4.8.

As expected, the phosphorylation of STAT1 at Ser727 was increased by IFN-γ over the time course, with the maximal level of expression observed at 3h. Phosphorylation at Tyr701 was also induced dramatically and declined after reaching its highest level at the 30min time point. Thus, the overall pattern of

STAT1 phosphorylation by IFN-γ in Hep3B cells was similar to that observed in THP-1 macrophages (Figure 3.9). The Hep3B cell line therefore provides a valuable cellular model system for studying IFN-γ-regulated STAT1 activation.

The effect of PD98059 on STAT1 phosphorylation in Hep3B cells was also investigated by western blot analysis. As for THP-1 macrophages, the cells were treated with PD98059 at a series of concentrations (5, 30 and 50 μM) or DMSO followed by stimulation with IFN-γ (100U/ml) for 30min. PD98059 attenuated the IFN-γ-stimulated STAT1 phosphorylation at Ser727 in a concentration-dependent manner. The overall inhibition by PD98059 was comparable to that in macrophages. Unexpectedly, the phosphorylation at Tyr701, which was found not to be inhibited by either PD98059 or U0126 in THP-1 cells, was reduced by pre-treatment of Hep3B cells with PD98059 at a higher concentration of 50μM (Figure 4.9). This is, however, consistent with what was observed in human primary macrophages, where STAT1 phosphorylation at both sites was inhibited by pre-treatment with PD98059 (Figure 4.7). Therefore, in addition to the Ser727 site found in THP-1 cells, ERK might also be involved in regulating STAT1 Tyr701 phosphorylation in hepatocytes. Given that STAT1 activity is regulated by phosphorylation at both sites, the Hep3B cell line, with its high transfection efficiency, provides a useful cellular model system for studying the mechanisms underlying the regulation of STAT1 activity in response to IFN-γ as well as that for MCP-1 promoter activation as previously carried out in our laboratory (Harvey et al., 2007).

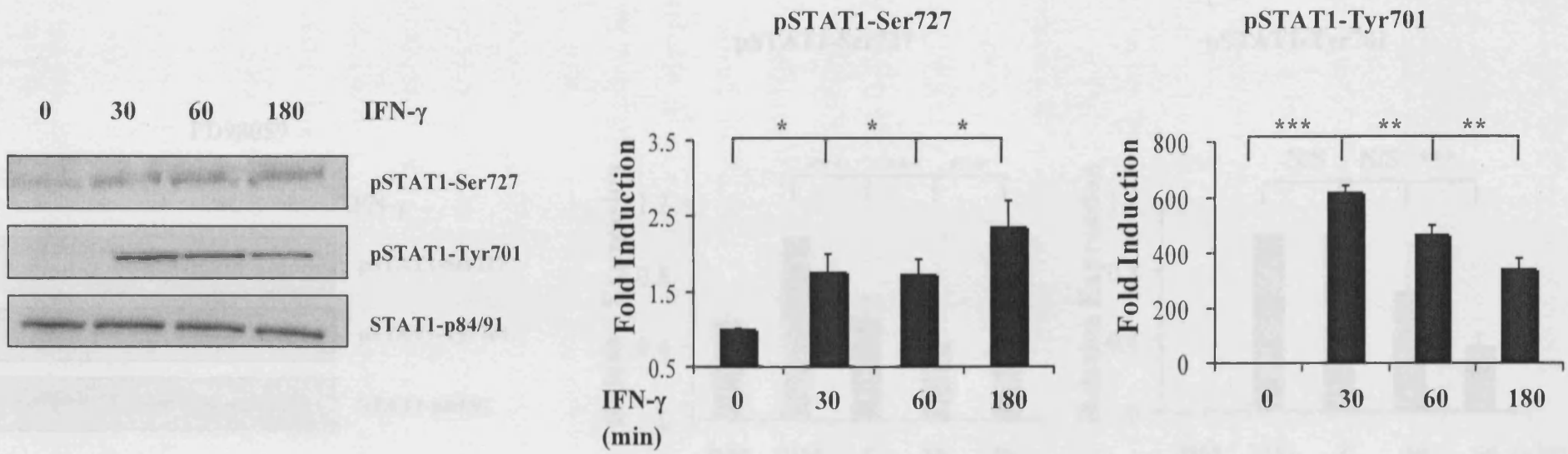


Figure 4.8 Effect of IFN-γ on the phosphorylation of STAT1 at Ser727 and Tyr701 over time in Hep3B cells

Hep3B cells were treated with IFN-γ for the indicated period of time and whole cell protein extracts were prepared. Western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the fold induction (mean ± SD) in the phosphorylation level of STAT1 at either of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis from three independent experimental series (the value for the 0min control point has been arbitrarily assigned as 1). Student's *t* test was carried out on IFN-γ-treated sample versus the 0min control (**P*<0.05; ***P*<0.01; ****P*<0.001).

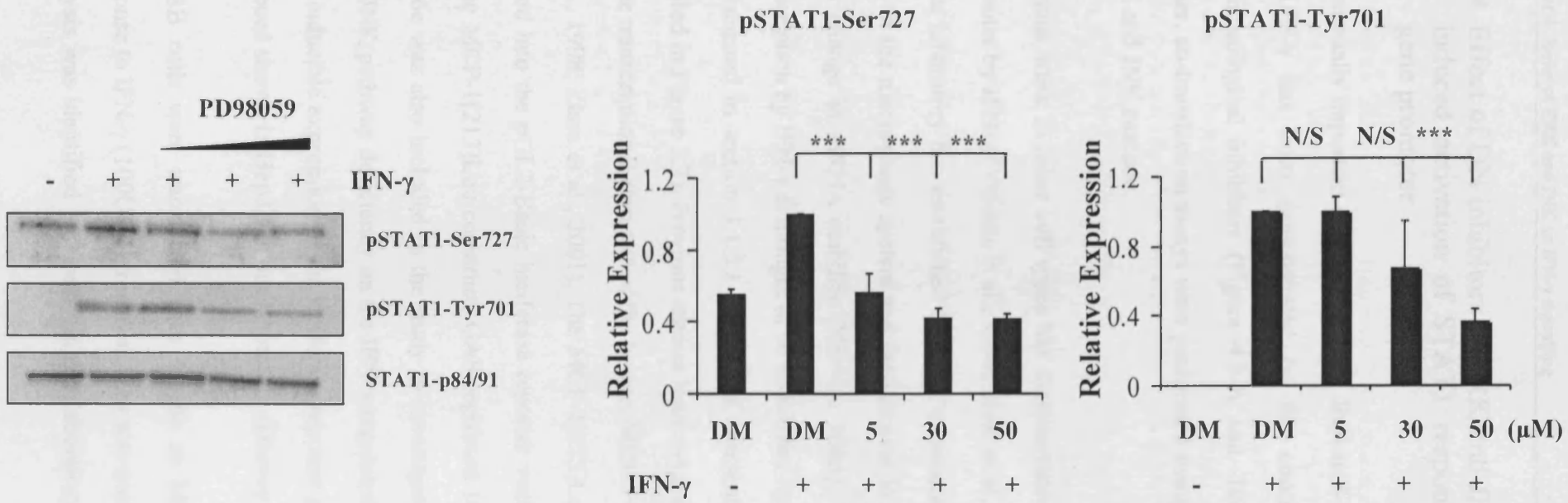


Figure 4.9 Effect of PD98059 on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701 in Hep3B cells

Hep3B cells were pre-treated with PD98059 (5, 30 and 50μM) as indicated or DMSO as a vehicle control. They were then cultured in the presence or the absence of IFN-γ for 30min. Whole cell protein extracts were prepared and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at either of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis from three independent experimental series (the value from IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Asterisks indicate significant attenuation by PD98059 of the IFN-γ-induced STAT1 phosphorylation observed in control cells (***P<0.001; N/S, not significant).

4.3.4 Effect of DN inhibitors of ERK and JNK pathways on the IFN-γ-induced activation of STAT1 responsive elements and MCP-1 gene promoter

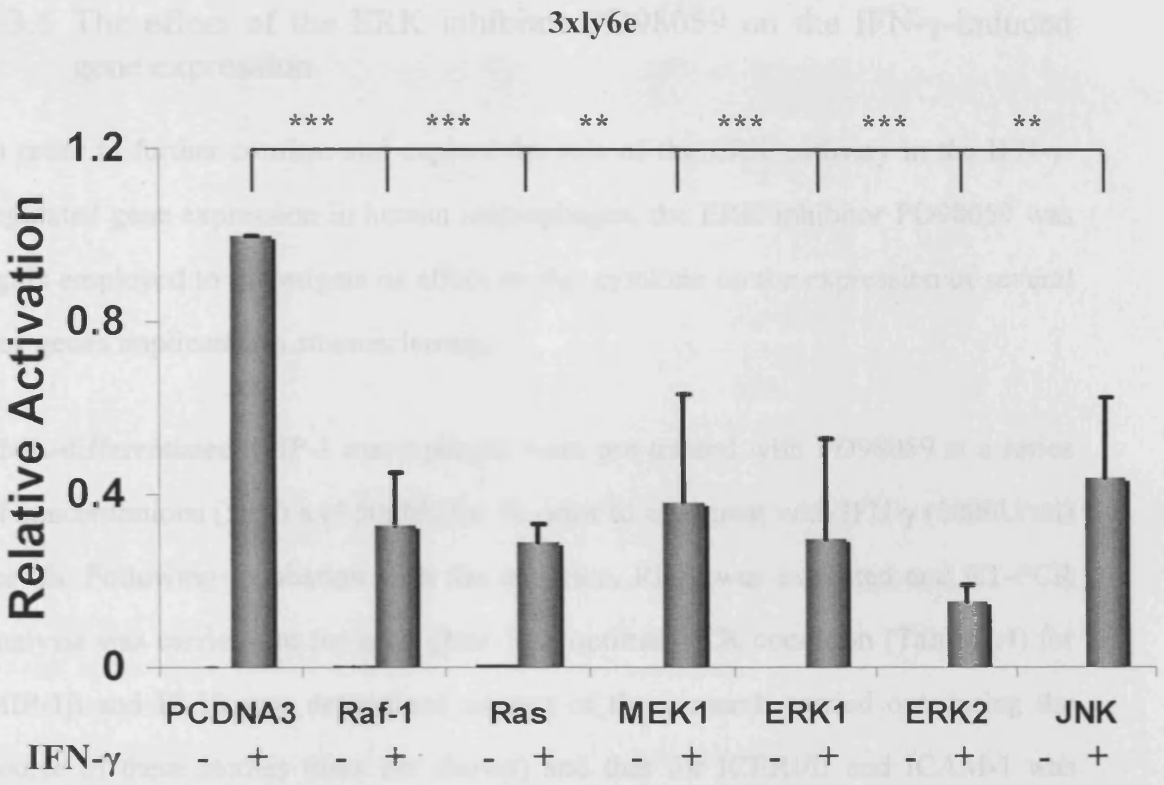
A potentially important role for ERK and JNK in STAT1 phosphorylation regulated by IFN-γ has been demonstrated by the concentration-dependent actions of pharmacological inhibitors (Figure 4.2-A and -B). In order to confirm the role further, co-transfection assays were performed using DN expression plasmids in the ERK and JNK cascade.

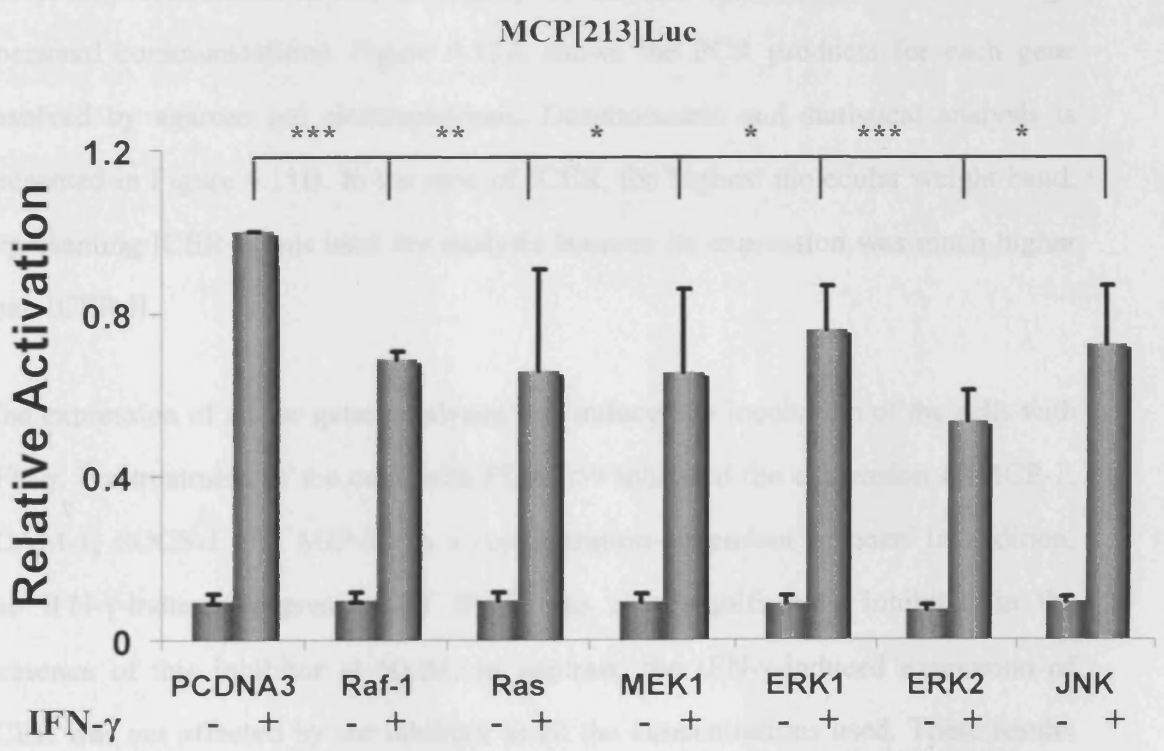
Previous work in other cell types has demonstrated activation of the MCP-1 gene promoter by IFN-γ (Valente et al., 1998; Zhou et al., 1998; Zhou et al., 2001). Work in our laboratory has established that IFN-γ increases the expression of the MCP-1 gene in the macrophage system and the increase in mRNA levels was not mediated by a change in mRNA stability (Harvey, 2006). The regulation of MCP-1 gene transcription by IFN-γ is thought to be controlled by a key proximal promoter region as discussed in section 3.1.5.1 (functional elements of the proximal promoter are detailed in Figure 3.2). Previous studies have mapped this region to 213bp upstream of the transcriptional start site of the human MCP-1 gene (Valente et al., 1998; Zhou et al., 1998; Zhou et al., 2001). The MCP-1[213]Luc construct contains this region cloned into the pGL2-Basic luciferase reporter vector (section 2.5.5.1). In addition to the MCP-1[213]Luc construct, GAS-regulated luciferase reporter gene plasmid, 3xly6e was also included in the study to investigate the consequences of the ERK and JNK pathway deficiency on the IFN-γ-regulated STAT1 transcriptional activity. The inducible expression of the luciferase reporter genes in both plasmids by IFN-γ has been shown in Hep3B in our laboratory (Harvey, 2006).

Hep3B cells were transfected with 3xly6e or MCP[213]Luc plasmids, and the response to IFN-γ (100U/ml) treatment (6h) was analysed (the optimal time point for analysis was identified previously in our laboratory (Harvey, 2006)). The reporter

construct was co-transfected with a plasmid specifying for a DN mutant for Ras, Raf-1, MEK1, ERK1, ERK2 or JNK with the empty plasmid vector pcDNA3 used as a control. DN forms of Ras and Raf-1 were included here as they represent important upstream components of the ERK pathway (Minden et al., 1994; Ramos, 2008). Ras has also been reported to contribute to JNK activation in response to some stimuli (Minden et al., 1994; Li et al., 1996; Auer et al., 1998). Following co-transfection and incubation in the absence or the presence of IFN-γ for 6h, the cells were harvested and extracts prepared for luciferase assay, and the protein concentration for each sample was determined (sections 2.5 and 2.6.5). Luciferase activity was then normalised to the protein concentration of each sample. We normalised the data to protein concentration and not to the activity of another internal control plasmid (e.g. CMV-β-galactosidase or RSV-β-galactosidase) because in our experience most of these promoters are also responsive to certain cytokines or extracellular mediators (Ali, 2007).

Results are presented in Figure 4.10 as the mean relative activity changes (±SD) (induction level in the presence of pcDNA3 assigned as 1). A student's *t* test was performed on the data and the inhibition in promoter activation by all these DN mutants was found to be significant, supporting the role of the ERK and JNK pathways in the IFN-γ-mediated regulation of STAT1-dependent gene transcription, including MCP-1.


3xly6e
***
***
**
***
***
**
1.2
0.8
0.4
0
Relative Activation
PCDNA3
Raf-1
Ras
MEK1
ERK1
ERK2
JNK
IFN-γ
- + - + - + - + - + - + - +


MCP[213]Luc
***
**
*
*
***
*
1.2
0.8
0.4
0
Relative Activation
PCDNA3
Raf-1
Ras
MEK1
ERK1
ERK2
JNK
IFN-γ
- + - + - + - + - + - + - +

4.3.5 The effect of the ERK inhibitor PD98059 on the IFN-γ-induced gene expression

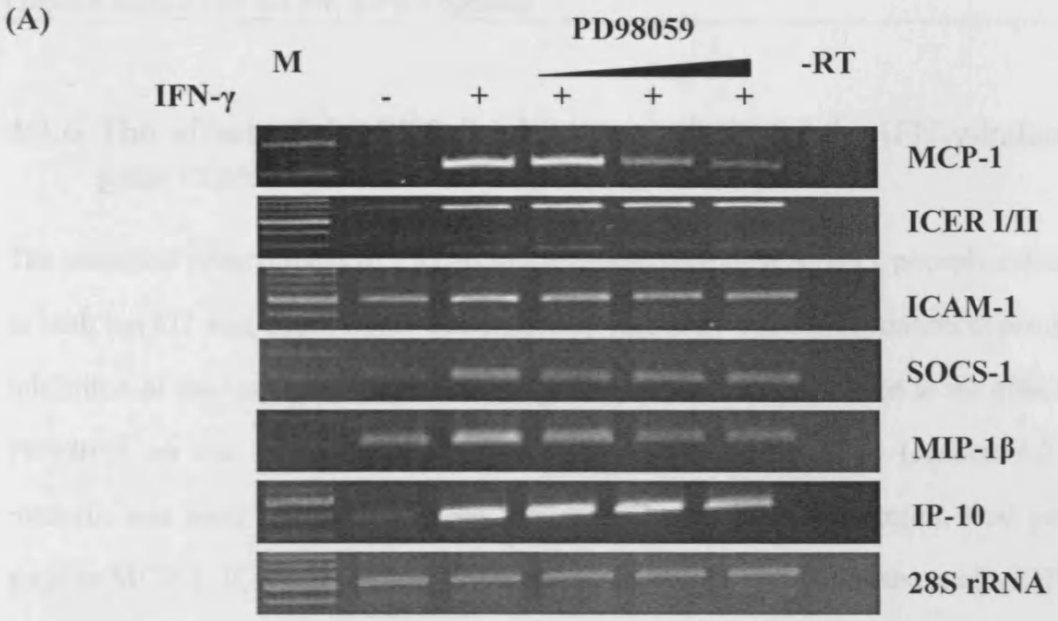
In order to further confirm and explore the role of the ERK pathway in the IFN-γ-regulated gene expression in human macrophages, the ERK inhibitor PD98059 was again employed to investigate its effect on this cytokine on the expression of several key genes implicated in atherosclerosis.

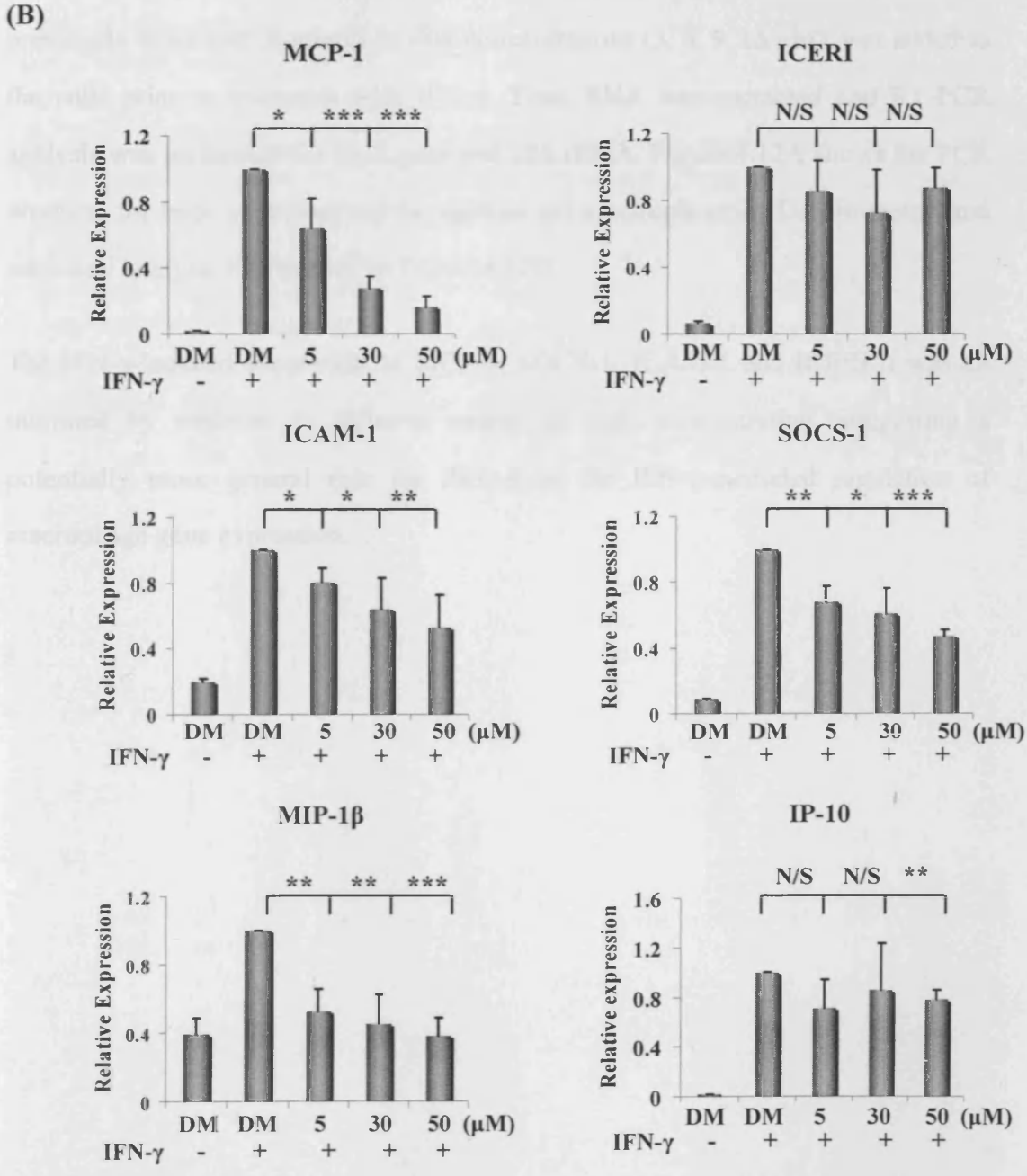
PMA-differentiated THP-1 macrophages were pre-treated with PD98059 at a series of concentrations (5, 30 and 50μM) for 1h prior to treatment with IFN-γ (1000U/ml) for 3h. Following incubation with the cytokine, RNA was extracted and RT-PCR analysis was carried out for each gene. The optimal PCR condition (Table 2.4) for MIP-1β and IP-10 was determined as part of the research carried out during the course of these studies (data not shown) and that for ICERI/II and ICAM-1 was previously determined in our laboratory by E. Harvey (2006) and Nishi Singh (personal communication). Figure 4.11A shows the PCR products for each gene resolved by agarose gel electrophoresis. Densitometric and statistical analysis is presented in Figure 4.11B. In the case of ICER, the highest molecular weight band, representing ICER I, was used for analysis because its expression was much higher than ICER II.

The expression of all the genes analysed was induced by incubation of the cells with IFN-γ. Pre-treatment of the cells with PD98059 inhibited the expression of MCP-1, ICAM-1, SOCS-1 and MIP-1β in a concentration-dependent manner. In addition, the IFN-γ-induced expression of IP-10 was also significantly inhibited in the presence of this inhibitor at 50μM. In contrast, the IFN-γ-induced expression of ICER was not affected by the inhibitor at all the concentrations used. These results therefore suggest that the ERK pathway plays a major, but relatively selective role, in the regulation of IFN-γ-inducible gene expression in human macrophages.

Figure 4.11 Effect of PD98059 on the IFN-γ-induced expression of MCP-1, ICERI/II, ICAM-1, SOCS-1, MIP-1β and IP-10 genes

PMA-differentiated THP-1 cells were pre-treated with PD98059 (5, 30 and 50μM) or DMSO as a vehicle control. They were then cultured in the presence or the absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1, ICER, ICAM-1, SOCS-1, MIP-1β, IP-10 and 28S rRNA. The amplification products were analysed by agarose gel electrophoresis (A). The images shown are representative of three independent experiments. M denotes the 100bp DNA ladder (NEB) (see Appendix I) and –RT shows a reaction in which no reverse transcriptase was included for the cDNA synthesis step (RNA from cells incubated with DMSO and IFN-γ was used). Panel B shows the relative expression (mean ±SD) of the indicated gene normalised to the expression of 28S rRNA as determined by densitometric analysis from three independent experimental series (IFN-γ-treated, DMSO control assigned as 1). Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ-treated control and samples incubated with PD98059 and IFN-γ (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; N/S, not significant).


(A)
PD98059
M
-RT
IFN-γ - + + + +
MCP-1
ICER I/II
ICAM-1
SOCS-1
MIP-1β
IP-10
28S rRNA


(B)
MCP-1
* *** ***
Relative Expression
1.2
0.8
0.4
0
DM DM 5 30 50 (μM)
IFN-γ - + + + +
ICERI
N/S N/S N/S
ICAM-1
* * **
SOCS-1
** * ***
MIP-1β
** ** ***
IP-10
N/S N/S **
Relative expression
1.6

4.3.6 The effect of the PKC-δ inhibitor rottlerin on the IFN-γ-induced gene expression

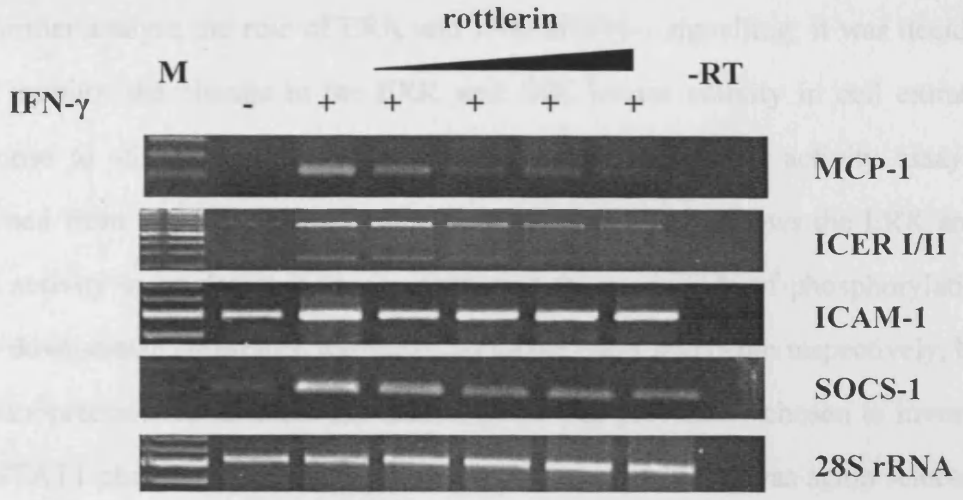
The potential involvement of PKC-δ in the IFN-γ-regulated STAT1 phosphorylation at both Ser727 and Tyr701 was demonstrated earlier by the concentration dependent inhibition of the response by rottlerin (Figure 4.4A). As a comparison to the effect of PD98059 on the IFN-γ-mediated regulation of gene expression (section 4.3.5), rottlerin was used to investigate the role of PKC-δ in such regulation. Key genes such as MCP-1, ICAM-1, SOCS-1 and ICER I/II were selected for the study. THP-1 cells were prepared and treated with the inhibitor (or DMSO) and the cytokine as previously described. Rottlerin, at four concentrations (3, 6, 9, 15 μM), was added to the cells prior to treatment with IFN-γ. Total RNA was extracted and RT-PCR analysis was performed for each gene and 28S rRNA. Figure 4.12A shows the PCR products for each gene resolved by agarose gel electrophoresis. Densitometric and statistical analysis is presented in Figure 4.12B.

The IFN-γ-induced expression of MCP-1, SOCS-1, ICAM-1 and ICERI/II was all inhibited by rottlerin, to different extent, at each concentration, suggesting a potentially more general role for PKC-δ in the IFN-γ-mediated regulation of macrophage gene expression.

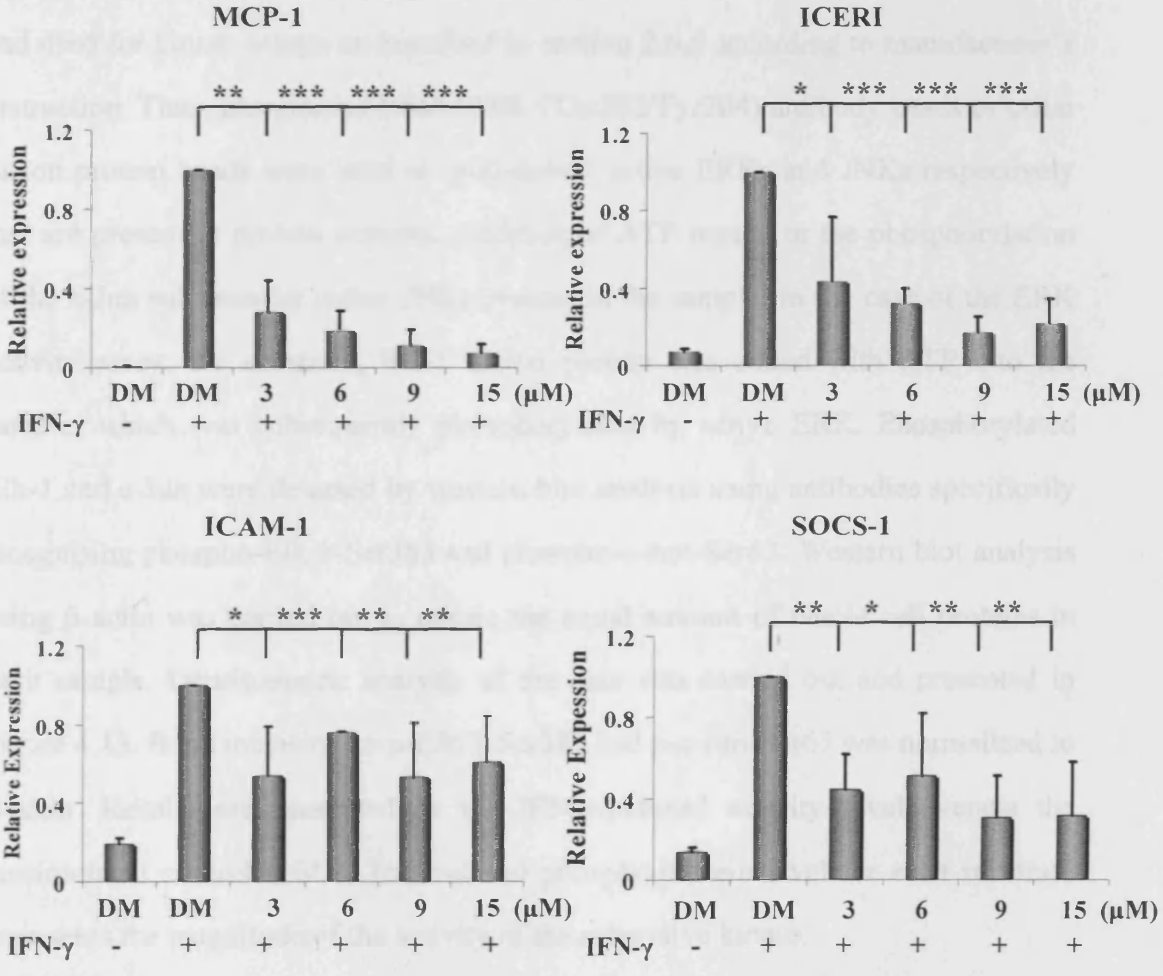
Figure 4.12 The effect of rottlerin on the IFN-γ-induced expression of MCP-1, ICERI/II, ICAM-1 and SOCS-1 genes

PMA-differentiated THP-1 cells were pre-treated with rottlerin (3, 6, 9 and 15μM) or DMSO as a vehicle control. They were then cultured in the presence or the absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1, ICER, ICAM-1, SOCS-1 and 28S rRNA. The amplification products were analysed by agarose gel electrophoresis (A). The images shown are representative of three independent experiments. M denotes the 100bp DNA ladder (NEB) and –RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (RNA from cells incubated with DMSO and IFN-γ was used). Panel B shows the relative expression (mean ±SD) of the indicated gene normalised to the expression of 28S rRNA as determined by densitometric analysis from three independent experimental series (IFN-γ-treated, DMSO control assigned as 1). Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with rottlerin and IFN-γ (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

(A)


rottlerin
M
-RT
IFN-γ
- + + + + +
MCP-1
ICER I/II
ICAM-1
SOCS-1
28S rRNA

(B)


MCP-1
ICERI
ICAM-1
SOCS-1
Relative expression
Relative Expression
DM DM 3 6 9 15 (μM)
IFN-γ - + + + + +

4.3.7 Induction of ERK and JNK activity by IFN-γ

To further analyse the role of ERK and JNK in IFN-γ signalling, it was decided to next monitor the change in the ERK and JNK kinase activity in cell extracts in response to stimulation of the cells with IFN-γ. The kinase activity assay kits, obtained from Cell Signalling Technology (section 2.6.6), allows the ERK and the JNK activity to be determined via measuring the magnitude of phosphorylation of their downstream substrates, transcription factor Elk-1 and c-Jun respectively, by the immunoprecipitated kinases. The 30min point was previously chosen to investigate the STAT1 phosphorylation in response to IFN-γ, and hence was again selected for analysing the ERK and JNK activities.

THP-1 cells were treated with IFN-γ for 0 and 30min. Cell lysates were prepared and used for kinase assays as described in section 2.6.6 according to manufacturer's instruction. Thus, phospho-p42/44/MAPK (Thr202/Tyr204) antibody beads or c-Jun fusion protein beads were used to 'pull-down' active ERKs and JNKs respectively that are present in protein extracts. Addition of ATP results in the phosphorylation of the c-Jun substrate by active JNKs present in the sample. In the case of the ERK activity assay, the substrate, Elk-1 fusion protein was added with ATP into the sample, which was subsequently phosphorylated by active ERK. Phosphorylated Elk-1 and c-Jun were detected by western blot analysis using antibodies specifically recognising phospho-Elk-1-Ser383 and phospho-c-Jun-Ser63. Western blot analysis using β-actin was carried out to ensure the equal amount of whole cell proteins in each sample. Densitometric analysis of the data was carried out and presented in Figure 4.13. Band intensity for p-Elk-1-Ser383 and p-c-Jun-Ser63 was normalized to β-actin. Results are presented as the IFN-γ-induced activity levels versus the unstimulated control (±SD). The induced phospho-protein level for each substrate represents the magnitude of the activity of the respective kinase.

As shown in Figure 4.13, the cells had low constitutive activity of both kinases in the absence of external IFN-γ. The activity of ERK was induced almost 3 fold following stimulation of the cells with IFN-γ for 30min whereas the magnitude of induction of JNK was about 1.5 fold. The effect of IFN-γ on the ERK activity was confirmed by a time-course experiment. THP-1 cells were treated with IFN-γ for 0, 15, 30, 60, 180 and 360min. Cell lysates were prepared and kinase assays were performed as discussed above. Figure 4.14 shows that the activity was induced by IFN-γ in a time-dependent manner and reached its highest level at 60min. The phospho-Elk1 levels seem at approximately similar levels until the end of the time course (6h), although an unusual decrease was observed at the 3h point. This could probably be due to a technical error as only a single experiment has been performed. Further experiments will be required to confirm this.

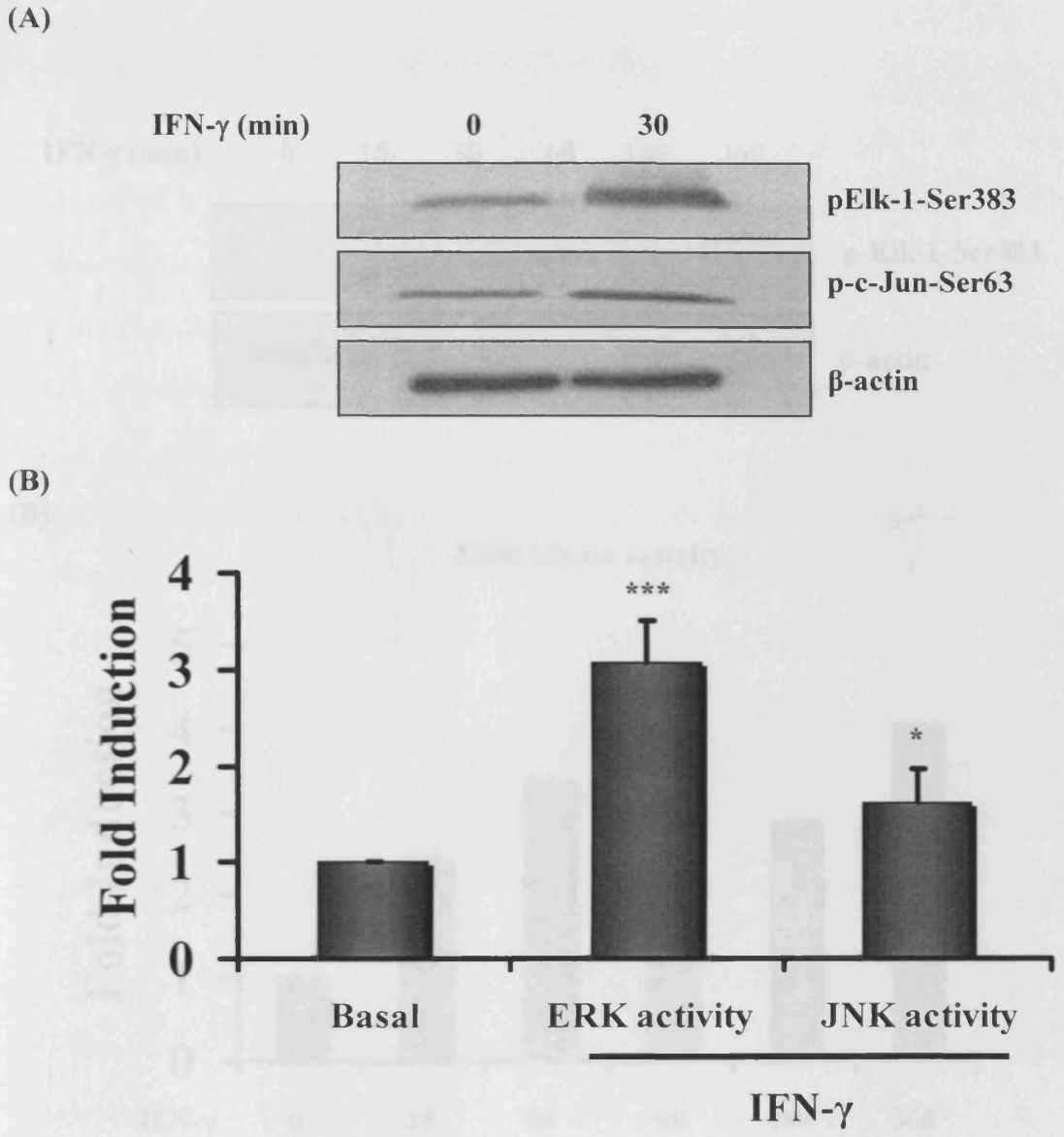
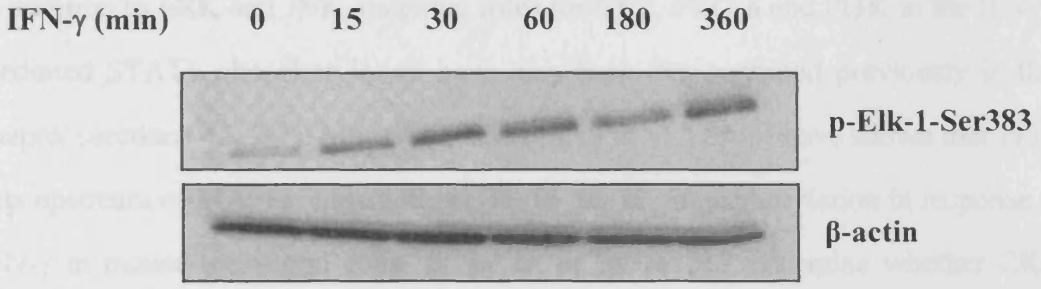


Figure 4.13 The effect of IFN-γ on ERK and JNK activities

PMA-differentiated THP-1 cells were treated with IFN-γ or left untreated for 30min and protein extracts were prepared (section 2.6.6). The ERK and JNK kinase assays were carried out using equal amount of proteins and the blotted membranes were probed with anti-phospho-Elk1 (Ser383) and anti-phospho-c-Jun (Ser63) antibodies as shown in panel A. For the analysis of β-actin levels, 5μg of protein extract for each sample was subjected to western blot analysis as described previously (Panel A). Panel B displays the ratio of phospho-Elk1:β-actin or phospho-c-Jun:β-actin, normalised to untreated control, which has been arbitrarily assigned as 1. The data shown in panel B is the mean (±SD) from three independent experimental series. The student's t test was performed on the IFN-γ-stimulated samples versus basal activity without treatment with the cytokine (***P<0.001; *P<0.05).

(A)



(B)

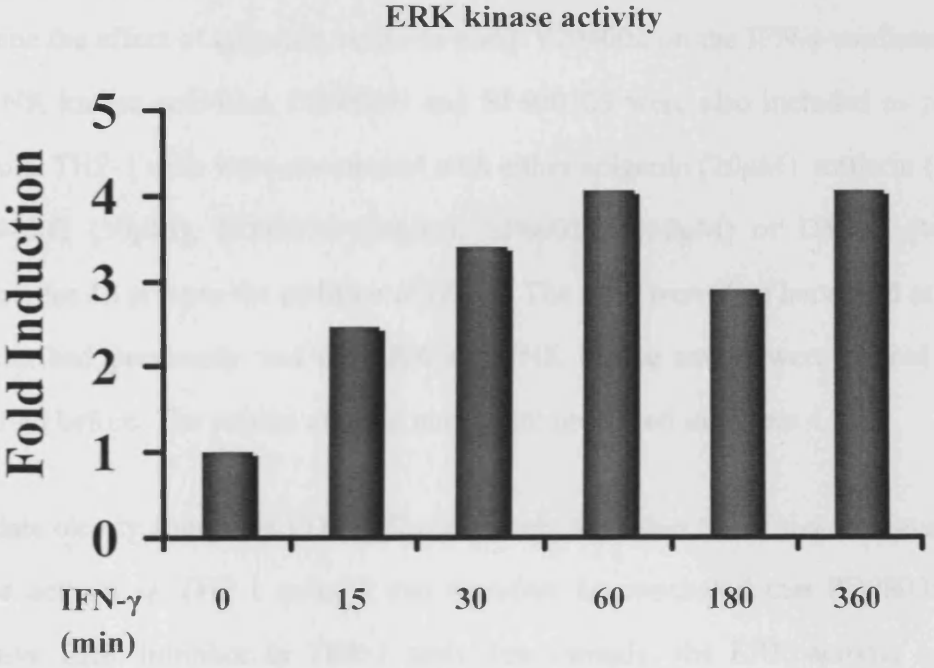


Figure 4.14 The effect of IFN-γ on the ERK activity over a time course

PMA-differentiated THP-1 cells were treated with IFN-γ for the indicated period of time and protein extracts were prepared (section 2.6.6). ERK kinase assays were carried out using an equal amount of protein at each time point and blotted membranes were probed with anti-phospho-Elk1 (Ser383) antibody as shown in panel A. For the analysis of β-actin levels, 5μg of protein extract for each sample was subjected to western blot analysis as described previously (Panel A). Panel B displays the ratio of phospho-Elk-1:β-actin, normalised to the 0min control which has been assigned as 1. Data is obtained from a single experiment.

4.3.8 Effect of the various pharmacological inhibitors on the IFN-γ-induced ERK and JNK kinase activity

In addition to ERK and JNK, potential roles for CK2, PKC-δ and PI3K in the IFN-γ-mediated STAT1 phosphorylation have also been demonstrated previously in this chapter (sections 4.2.2-3). Moreover, Choudhury et al. (2004) have shown that PI3K acts upstream of MAPK in mediating STAT1 Ser727 phosphorylation in response to IFN-γ in mouse mesangial cells. It is thus of interest to determine whether CK2, PKC-δ or PI3K act upstream of ERK or JNK in mediating IFN-γ-stimulated responses in human macrophages. To investigate this possibility, it was decided to examine the effect of apigenin, rottlerin and LY294002 on the IFN-γ-mediated ERK and JNK kinase activities. PD98059 and SP600125 were also included as positive controls. THP-1 cells were pre-treated with either apigenin (20μM), rottlerin (10μM), LY294002 (30μM), PD98059 (50μM), SP600125 (30μM) or DMSO (vehicle-control) for 1h prior to the addition of IFN-γ. The cells were then harvested at 30min as described previously and the ERK and JNK kinase assays were carried out as specified before. The results of these assays are presented in Figure 4.15.

The data clearly show that PD98059 completely abolishes the IFN-γ-mediated ERK kinase activity in THP-1 cells. It can therefore be concluded that PD98059 is an effective ERK inhibitor in THP-1 cells. Interestingly, the ERK activity was not inhibited, but instead dramatically enhanced by treatment of the cells with SP600125, apigenin and LY294002. This suggests a potential cross-talk between the ERK pathway and JNK, CK2 and PI3K pathways. In the case of the IFN-γ-mediated JNK activity, SP600125 failed to significantly attenuate it. This could potentially be explained by the mode of action of SP600125, which acts as a competitor for ATP binding to JNK (Bennett et al., 2001). The addition of ATP molecules in the *in vitro* kinase assay probably unexpectedly overcame the competitive association of the inhibitor to the ATP-binding pocket, and thereby JNK regained its kinase activity. Moreover, the JNK activity was enhanced significantly by treatment of the cells

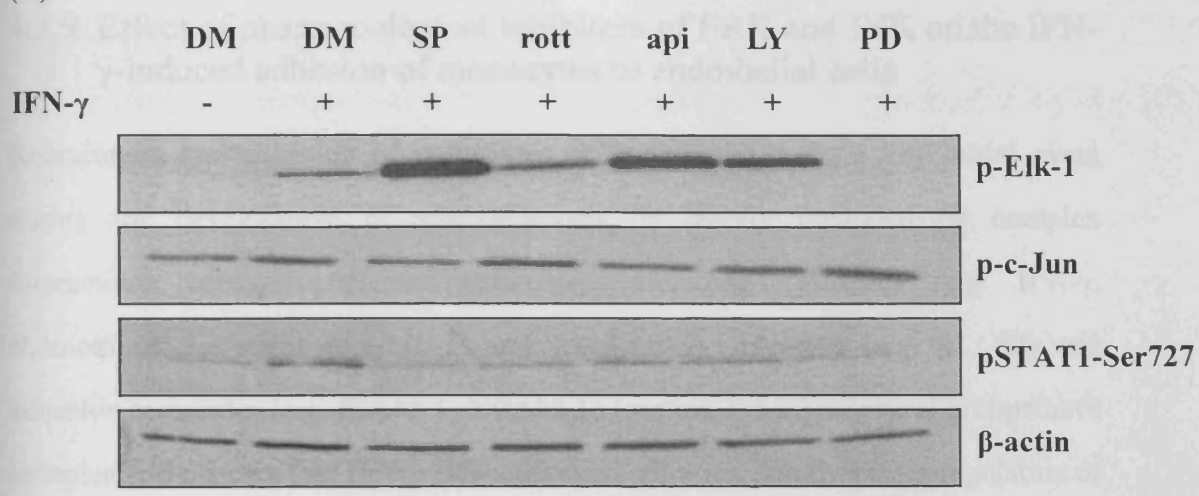
with PD98059, but not by apigenin, LY294002 or rottlerin, thereby again suggesting some sort of cross-talk between the ERK and JNK pathways. To rule out the possibility that the failure to suppress the kinase activity by some of these inhibitors was due to the lack of their inhibitory activity (e.g. due to storage), the effect of each inhibitor on STAT1 phosphorylation at Ser727 was analysed, and a similar extent of suppression was observed as demonstrated before (Figures 4.2 and 4.4).

Cell viability was not impaired by each inhibitor according to the trypan blue exclusion assay (Table 4.2), and hence the changes seen in kinase activity were not attributable to cell death. Also of note, the β-actin protein levels did not vary in the presence of these inhibitors, thereby strongly indicating that the observed effect on the ERK and JNK activity was not due to a global change in the cellular protein levels by the inhibitors.

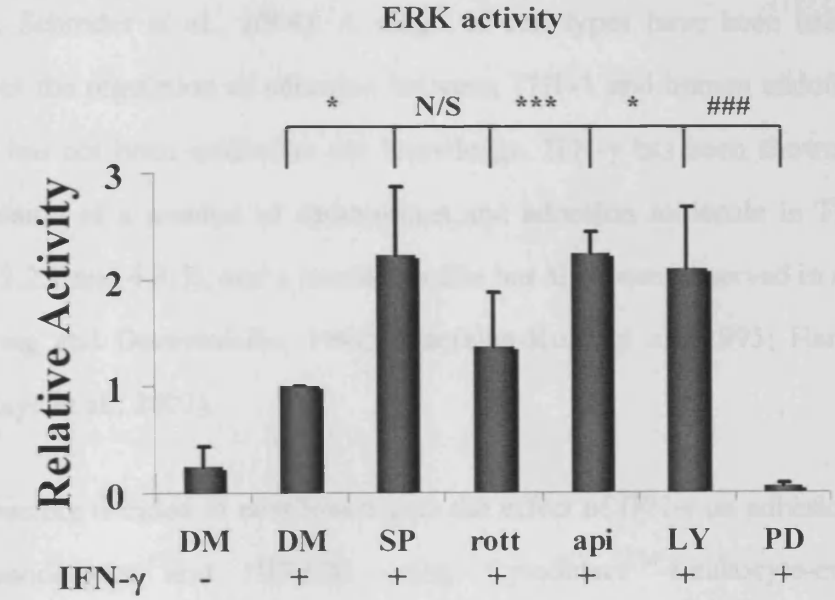
Figure 4.15 The effect of inhibitors on the IFN-γ-induced ERK and JNK activities

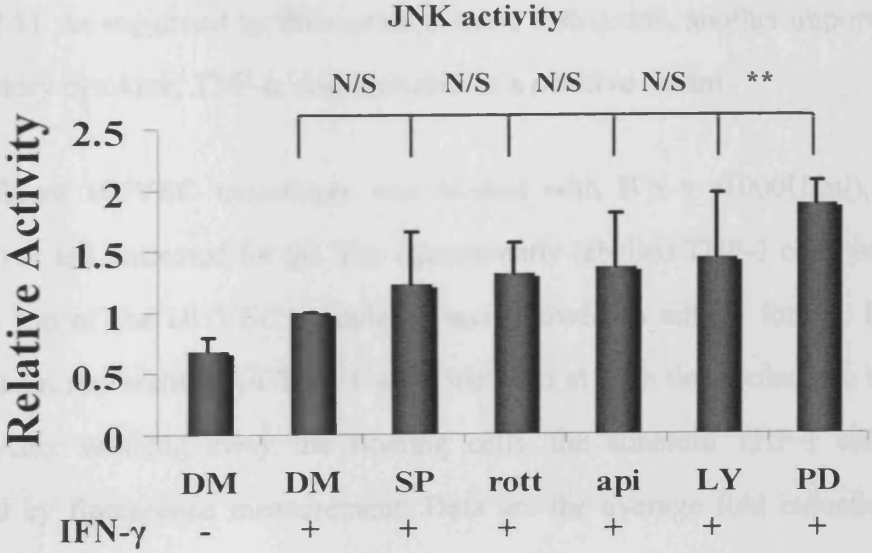
PMA-differentiated THP-1 cells were pre-treated with each of the specific inhibitors or DMSO as a vehicle control for 1h prior to the addition of IFN-γ for 30min. Protein extracts were prepared and the ERK and JNK kinase assays and β-actin westerns were carried out as described in Figure 4.15 (A). For the analysis of phospho-STAT1-Ser727 levels, 10μg of protein extract for each sample was subjected to western blot analysis as described previously (panel A). Panel B shows the ratio of phospho-Elk1:β-actin and phospho-c-Jun: β-actin, normalised to the IFN-γ treated DMSO control (assigned as 1). The data shown in panel B is the mean (±SD) from three independent experimental series. Student's t test was carried out on samples treated with each inhibitor versus the IFN-γ-treated, DMSO control sample. Significance of induction (***P<0.001; **P<0.01; *P<0.05) and significance of inhibition (###P<0.001; N/S not significant) is shown. Abbreviations: DM, DMSO; PD, PD98059; SP, SP600125; api, apigenin; LY, LY294002; rott, rottlerin.

(A)


DM
DM
SP
rott
api
LY
PD
IFN-γ
- + + + + + +
p-Elk-1
p-c-Jun
pSTAT1-Ser727
β-actin

(B)


ERK activity
* N/S *** * ###
Relative Activity
3
2
1
0
DM DM SP rott api LY PD
IFN-γ - + + + + + +


JNK activity
N/S N/S N/S N/S **
Relative Activity
2.5
2
1.5
1
0.5
0
DM DM SP rott api LY PD
IFN-γ - + + + + + +

4.3.9 Effect of pharmacological inhibitors of ERK and JNK on the IFN-γ-induced adhesion of monocytes to endothelial cells

Recruitment and adhesion of monocytes to endothelial cells, a key initial event during the development of atherosclerosis, is largely mediated by complex interactions between different molecules, including cytokines (e.g. IFN-γ, chemokines), selectins (e.g. L, P and E-selectins), integrins (e.g. β1, β2) and adhesion molecules (e.g. ICAM-1, VCAM-1) (section 1.4.2.3). Several groups have independently shown that IFN-γ promotes such adhesion largely via up-regulation of expression of these key molecules (Wang et al., 1994; Chang et al., 2002; Navarro et al., 2003; Schroder et al., 2004). A range of cell types have been used in these studies, but the regulation of adhesion between THP-1 and human endothelial cells by IFN-γ has not been studied to our knowledge. IFN-γ has been shown to induce the expression of a number of chemokines and adhesion molecule in THP-1 cells (sections 3.2.1 and 4.3.5), and a similar profile has also been observed in endothelial cells (Wong and Dorovini-Zis, 1992; Marfaing-Koka et al., 1995; Harvey et al., 2007; Kitaya et al., 2007).

It was therefore decided to next investigate the effect of IFN-γ on adhesion between THP-1 monocytes and HUVEC using CytoSelect^{TM}-Leukocyte-endothelium adhesion assay kit according to the manufacturer's protocol (Cell BioLabs Inc.) (section 2.8). As suggested by the manufacturer's instruction, another important pro-inflammatory cytokine, TNF-α, was included as a positive control.

The confluent HUVEC monolayer was treated with IFN-γ (1000U/ml), TNF-α (50ng/ml) or left untreated for 6h. The fluorescently-labelled THP-1 cells were then added on top of the HUVEC monolayer and allowed to adhere for 1h. HUVEC alone without any addition of THP-1 were included at each time point as a negative control. After washing away the floating cells, the adherent THP-1 cells were quantified by florescence measurement. Data are the average fold induction from

two independent experiments and presented as the IFN-γ-induced adhesion levels normalized to unstimulated controls. As shown in Figure 4.16, addition of IFN-γ or TNF-α resulted in no induction in the adhesion between THP-1 and HUVEC at the 6h point.

U937 is another human monocytic cell line routinely in use in our laboratory (Harvey et al., 2007; Harris et al., 2008) and previous work has shown similar responses in THP-1 and U937 cells in response to IFN-γ actions (Harvey et al., 2007). U937 has also been successfully employed in similar adhesion assays performed by different groups (Weber et al., 1994; Weber et al., 1995; Marx et al., 1999; Chang et al., 2002). It was therefore decided to use U937 as a cell model to investigate the effect of IFN-γ on adhesion between monocytes and HUVEC.

4.3.9.1 IFN-γ-induced adhesion between U937 and HUVEC

The confluent HUVEC monolayer was treated with IFN-γ (1000U/ml), TNF-α (50ng/ml) or left untreated for the appropriate period of time as indicated in Figure 4.17. The fluorescently-labelled U937 cells were then added on top of the HUVEC monolayer and allowed to adhere for 1h. HUVEC alone without any addition of U937 were also included at each time point as a negative control. After washing away the floating cells, the adherent U937 cells were quantified by florescence measurement. Data are the mean (±SD) from three independent experiments and presented as the IFN-γ-induced adhesion levels normalised to the unstimulated control.

The data in Figure 4.17 show that addition of TNF-α result in approximately 3.4- and 6-fold induction of adhesion between U937 and HUVEC at 6h and 12h respectively, time points at which IFN-γ showed minimal effects. The adhesion level gradually increased in the presence of IFN-γ throughout the subsequent time points to reach maximal levels (about 3 fold induction) at 30h. This time point was

therefore selected for future studies. The U937 adhesion decreased marginally 2days after stimulation with IFN-γ, which is consistent with previous findings between human primary monocytes and HUVEC (Wang et al., 1994).

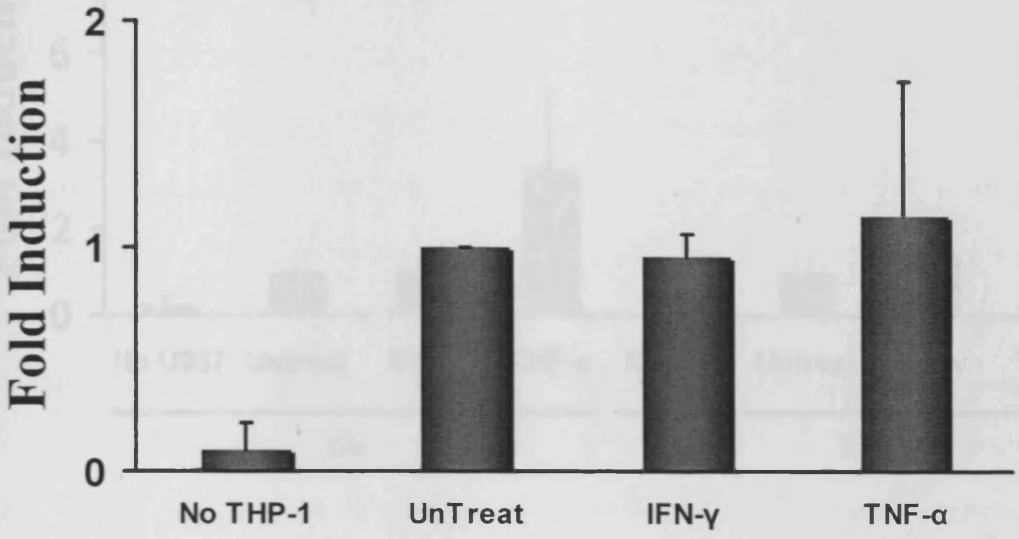


Figure 4.16 The effect of IFN-γ and TNF-α on adhesion between THP-1 and HUVEC at the 6h time point

Monolayers of HUVEC were treated with IFN-γ (1000U/ml) or TNF-α (50ng/ml) for 6h and LeukoTracker™-labelled THP-1 cells were added and allowed to adhere for 1h. The adherent cells were lysed and quantified by fluorescence measurement at 485nm/520nm. HUVEC monolayer without the addition of THP-1 cells was used as a negative control. The graphs show the average fold induction of adhesion by cytokines at each time point from 2 independent experiments (untreated control assigned as 1).

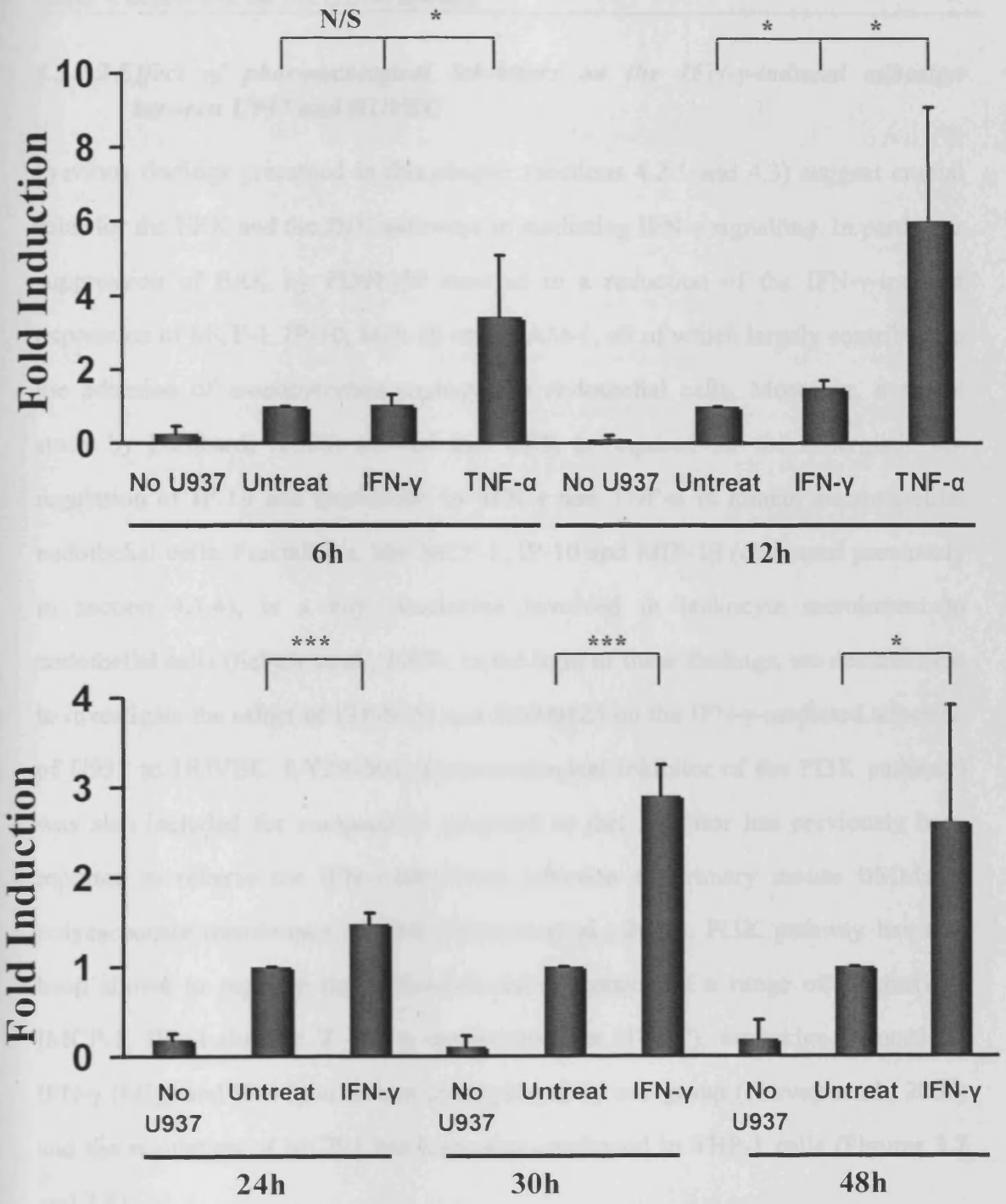


Figure 4.17 The effect of IFN-γ and TNF-α on adhesion between U937 and HUVEC over time

Monolayers of HUVEC were treated with IFN-γ (1000U/ml) or TNF-α (50ng/ml) for the indicated period of time and LeukoTracker™-labelled U937 cells were added and allowed to adhere for 1h. The adherent cells were lysed and quantified by fluorescence measurement at 485nm/520nm. HUVEC monolayer without the addition of U937 cells was used as a negative control. The graphs show the mean fold induction (±SD) of adhesion by cytokines at each time point from 3 independent experiments (untreated control assigned as 1). Student's *t* test was carried out on samples treated with cytokine versus untreated samples at each time point (***$P<0.001$; **$P<0.01$; *$P<0.05$; N/S, not significant).

4.3.9.2 Effect of pharmacological inhibitors on the IFN-γ-induced adhesion between U937 and HUVEC

Previous findings presented in this chapter (sections 4.2.1 and 4.3) suggest crucial roles for the ERK and the JNK pathways in mediating IFN-γ signalling. In particular, suppression of ERK by PD98059 resulted in a reduction of the IFN-γ-induced expression of MCP-1, IP-10, MIP-1β and ICAM-1, all of which largely contribute to the adhesion of monocytes/macrophages to endothelial cells. Moreover, a recent study by Lombardi (2008) showed that ERK is required for the synergistic up-regulation of IP-10 and fractalkine by IFN-γ and TNF-α in human microvascular endothelial cells. Fractalkine, like MCP-1, IP-10 and MIP-1β (discussed previously in section 4.1.4), is a key chemokine involved in leukocyte recruitment to endothelial cells (Schulz et al., 2007). In the light of these findings, we decided next to investigate the effect of PD98059 and SP600125 on the IFN-γ-mediated adhesion of U937 to HUVEC. LY294002 (pharmacological inhibitor of the PI3K pathway) was also included for comparative purposes as this inhibitor has previously been reported to reverse the IFN-γ-stimulated adhesion of primary mouse BMMs to polycarbonate membranes *in vitro* (Navarro et al., 2003). PI3K pathway has also been shown to regulate the IFN-γ-induced expression of a range of chemokines [MCP-1, IFN-inducible T cell α chemoattractant (ITAC), monokine induced by IFN-γ (Mig) and IP-10] in mouse macrophages by our group (Harvey et al., 2007) and the regulation of MCP-1 has been also confirmed in THP-1 cells (Figures 3.7 and 3.8).

Monolayers of confluent HUVEC were treated with the individual inhibitor or DMSO for 1h prior to further incubation with or without IFN-γ for 30h. The results from the ERK and JNK kinase assays (Figure 4.15) show that the repression of either ERK or JNK activity by the respective inhibitor resulted in the induced kinase activity of another, and hence the enhanced alternative kinase activity could potentially mask the impact of individual inhibitor on cellular adhesion. We

therefore decided to also include a combination of two inhibitors and analyse their joint effects on the IFN-γ-mediated monocyte/endothelial cell adhesion. Concentrations of 30μM for PD98059 and 20μM for SP600125 were initially selected because these were the concentrations used in human primary monocyte-derived macrophages and produced negligible cell death (Sesction 4.3.2). LY294002 was used at 10μM as this was the concentration previously employed by Navarro et al. (2003). The adhesion assay was performed as described before and the results are shown in Figure 4.18. Adhesion level in each sample was presented as a percentage of that seen in DMSO and IFN-γ-treated control cells. The cell viability of HUVEC was also determined using the ViaLight assay.

The inhibitors SP600125 and LY294002 attenuated the IFN-γ-stimulated adhesion between U937 and HUVEC by approximately 20% and 30% respectively. The addition of PD98059 however, resulted in a dramatic induction of adhesion by about 70% compared to DMSO and IFN-γ-treated control. As suggested previously, the induced adhesion could potentially be due to the enhanced JNK activity in the presence of PD98059 (Figure 4.15). These results in isolation suggest that the JNK and PI3K pathways potentially regulate the IFN-γ-stimulated adhesion of monocytes to endothelial cells. The exact role of ERK in the IFN-γ-induced adhesion remains unclear at present. However, pre-treatment of cells with PD98059 and SP600125 together resulted in more reduction in adhesion (45%) than that by SP600125 alone (20%), thereby suggesting a potential involvement of ERK in the IFN-γ-induced adhesion of monocytes to endothelial cells.

It is noteworthy that the ViaLight assay carried out on HUVEC (presented as green bars in Figure 4.18) showed that incubation with the inhibitor and cytokine for 31h (treatment with inhibitor for 1h plus with IFN-γ for another 30h) resulted in HUVEC viability that was lower than the inhibition levels of adhesion (blue bars in Figure 4.18). In addition, the total amount of protein (pink bars in Figure 4.18) extracted

from HUVEC and U937 after variety of treatments was reduced, possibly because of either decreased HUVEC viability or reduced number of U937 adhering to the HUVEC monolayer. For comparative purpose, the relative protein amount of each sample was presented as a percentage to the IFN-γ-treated DMSO control (arbitrarily assigned as 100%). These results suggest that the low HUVEC viability could have contributed to the decreased adhesion of U937 to HUVEC caused by the inhibitor(s). However, although PD98059 seemed to cause as much HUVEC cell death as the other inhibitors, the adhesion between U937 and HUVEC was not reduced. It is also important to note that the fluorescent readings are from labelled-monocytes, which were incubated for only 1h with cytokine/inhibitor-treated HUVEC. It is therefore difficult to come to any firm conclusions on whether the reduced adhesion by the inhibitors could be attributed to their actions on specific pathways, or simply due to impaired HUVEC viability. It is worth noting that HUVEC viability and total amount of protein obtained from HUVEC and U937 cells was unaffected by treatment with IFN-γ alone (Figure 4.18). The results suggest that the induced adhesion of IFN-γ-treated cells compared to the unstimulated samples was not due to any toxicity of the cytokine.

The high proportion of cell death caused by the inhibitors was probably because of the prolonged incubation period (31h). We thus next decided to investigate the effect of these inhibitors on the IFN-γ-induced U937 adhesion at much lower concentrations and analyse them in a concentration-dependent manner. Another ERK inhibitor, U0126 was also included here to provide comparison with PD98059. Figure 4.19 shows that with the exception of PD98059, the other three inhibitors (U0126, SP600125 and LY294002) attenuated the IFN-γ-stimulated adhesion of U937 to HUVEC, in a concentration-dependent manner. All these inhibitors at their highest concentrations reduced the adhesion by approximately 40% (presented as blue bars). However, the ViaLight assay showed that incubation with the inhibitor again resulted in low cell viability (presented as a black line in Figure 4.19).

Interestingly, incubation of the cells with PD98059 produced contrasting results to U0126 on adhesion of U937 to HUVEC. The reason behind the opposing action of these two specific inhibitors of ERK remains unclear. Therefore, the results suggest that it is at present not possible to come to any firm conclusions on the role of each kinase analysed on the IFN-γ-induced adhesion of U937 to HUVEC. Employment of inhibitors might not be the best strategy here because of the prolonged incubation period. RNA interference (RNAi) using small interfering RNA (siRNA) form of each kinase might facilitate our study on the IFN-γ-mediated monocyte/endothelial cell adhesion in the future.

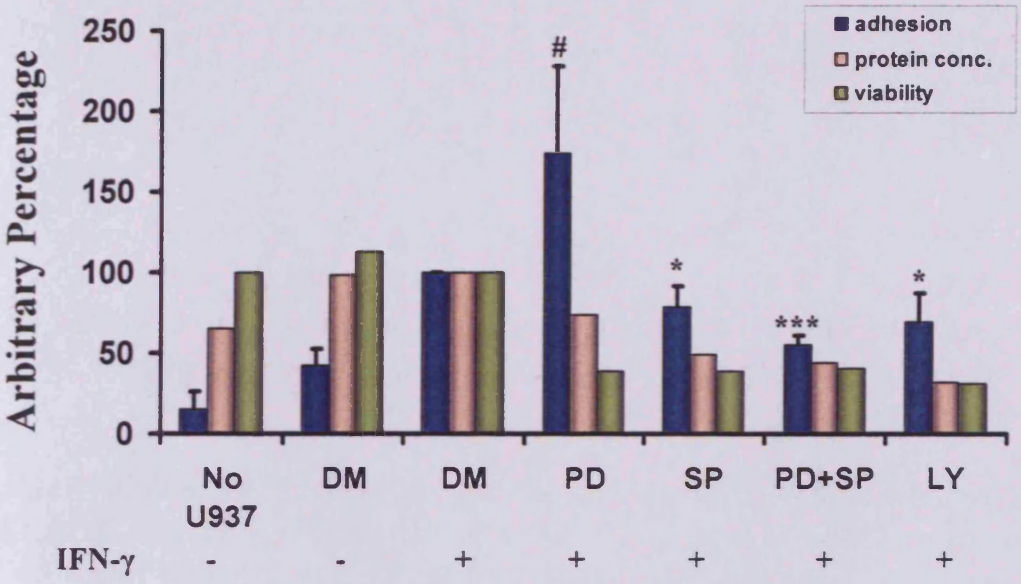
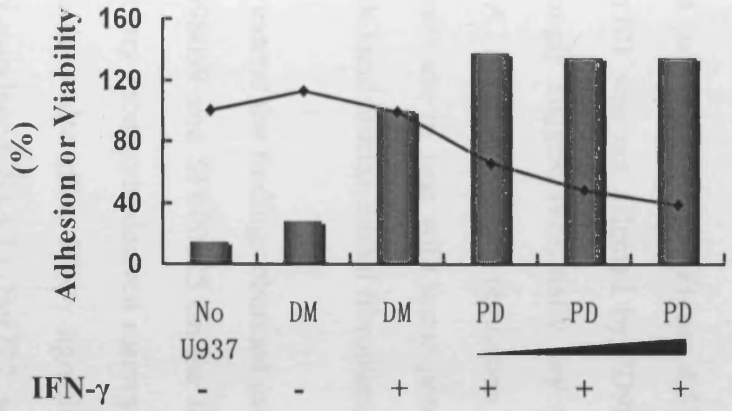


Figure 4.18 The effect of pharmacological inhibitors on the IFN-γ-induced adhesion between U937 and HUVEC at the 30h time point along with total amount of cellular proteins and viability of HUVEC cells under variety of treatments

Monolayers of HUVEC were pre-treated with PD98059 (30μM), SP600125 (20μM), LY294002 (10μM), combination of PD98059 (30μM) and SP600125 (20μM) or DMSO for 1h, and then incubated with IFN-γ (1000U/ml) for 30h. LeukoTracker™ - labelled U937 cells were added and allowed to adhere to HUVEC monolayers for 1h. Adherent cells were lysed and quantified by fluorescence measurement at 485nm/520nm. HUVEC monolayers without any treatment with the inhibitor or addition of U937 cells were used as negative controls. Blue bars in figures represent the average percentage (±SD) of adhesion under variety of treatments relative to DMSO (DM) plus IFN-γ-treated control (assigned as 100%), from four independent experiments. Student's *t* test was carried out on IFN-γ/inhibitor-treated samples versus IFN-γ-treated DMSO control sample (significance of induction: # $P<0.05$; significance of inhibition: * $P<0.05$, ** $P<0.01$). Pink bars in figure represent the total amount of cellular protein from HUVEC with adhered U937 monocytes under different treatment from a single experiment. The value from HUVEC with DMSO and IFN-γ-treated sample was arbitrarily assigned as 100%. Green bars in figure show the viability of HUVEC under different treatments analysed by the ViaLight assay (section 2.3.12.2). The value from HUVEC with DMSO and IFN-γ treated sample was assigned as 100% viable. Luminescence reading was obtained from a single experiment. Abbreviations: PD, PD98059; SP, SP600125; LY, LY294002; DM, DMSO

Figure 4.19 The effect of pharmacological inhibitors on the IFN-γ-induced adhesion between U937 and HUVEC at the 30h time point along with analysis of viability of HUVEC under variety of treatments

Monolayers of HUVEC were pre-treated with PD98059 (0.5, 1 and 3μM), U0126 (1, 2 and 4μM), SP600125 (0.5, 1 and 3μM), LY294002 (1, 2 and 5μM) or DMSO for 1h, and then incubated with IFN-γ (1000U/ml) for 30h. LeukoTracker™-labelled U937 cells were added and allowed to adhere to the HUVEC monolayer for 1h. Adherent cells were lysed and quantified by fluorescence measurement at 485nm/520nm. HUVEC monolayer without any treatment with the inhibitor or the addition of U937 cells was used as negative controls. Data were obtained from a single experiment. Blue bars in figures represent the percentage of adhesion under variety of treatments relative to DMSO plus IFN-γ-treated control (assigned as 100%). Black lines in figure show the viability of HUVEC under different treatments as determined using the ViaLight assay (section 2.3.12.2). The value from HUVEC with DMSO and IFN-γ treated sample was assigned as 100% viable. Luminescence reading was obtained from a single experiment. Abbreviations: PD, PD98059; SP, SP600125; LY, LY294002; DM, DMSO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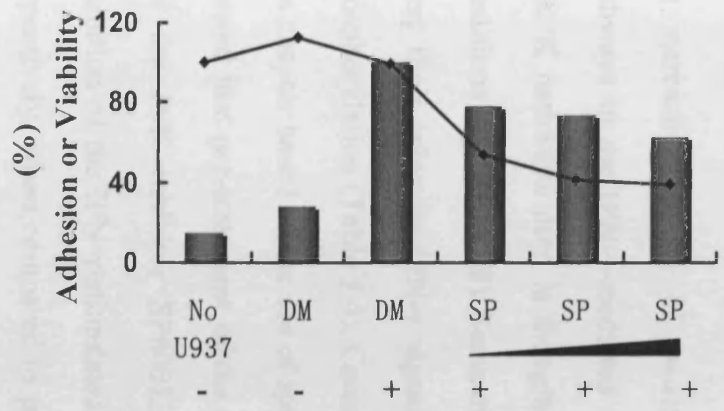
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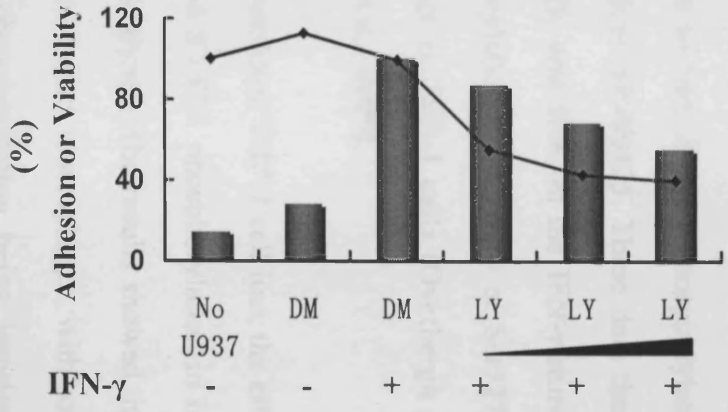
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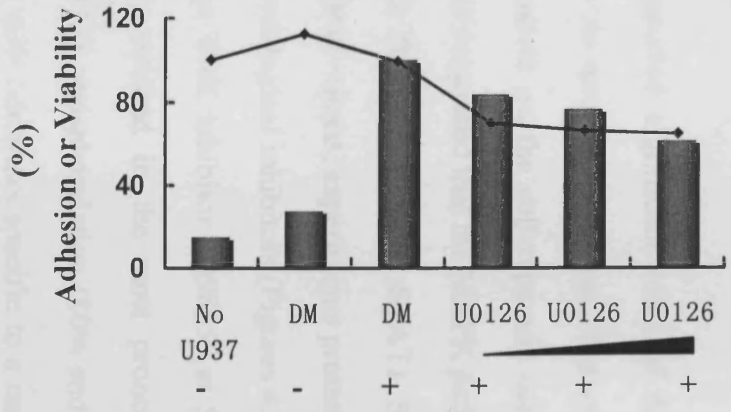
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4.4 Discussion

4.4.1 Roles of ERK and JNK in the IFN-γ-mediated regulation of gene expression in human macrophages

An increasing volume of evidence has supported significant roles of MAPK pathways in the IFN-γ-mediated signalling in an array of cell types. The specific MAPK pathways used is thought to be dependent on the cell type and culturing conditions (section 1.7.3). Some of these studies suggested that the MAPK pathways exert their roles in IFN-γ signalling through the regulation of STAT1 Ser727 phosphorylation (Table 1.4). Consistent with these reports, experiments presented in this chapter based on the use of specific pharmacological inhibitors (Figures 4.2-4.4) showed that pre-treatment of the cells with the ERK inhibitor PD98059 at 50μM, and the JNK inhibitor SP600125 at 30μM, resulted in the most pronounced inhibition of the IFN-γ-stimulated STAT1 Ser727 phosphorylation (80% and 73%, respectively) when compared to pre-treatment with inhibitors specific to a range of other candidate kinases, including CK2, PI3K, PKC-δ and CaMK II (Table 4.4). A potentially important role for ERK was again confirmed using another inhibitor of this pathway, U0126 (Figure 4.5). In contrast to this, STAT1 phosphorylation at Tyr701 was not affected by PD98059, U0126 or SP600125. These data therefore strongly suggest potentially key roles for ERK and JNK in the IFN-γ-stimulated STAT1 activation via regulation of phosphorylation specifically at Ser727. The results are in line with some previous findings in THP-1 cells (Overbergh et al., 2006) and murine demal fibroblasts (Yamana et al., 2009).

To extend the findings obtained using the differentiated THP-1 cell line, the effect of PD98059 and SP600125 on the IFN-γ-induced STAT1 phosphorylation in human primary monocyte-derived macrophages was analysed. The results showed that the inhibitors also affect IFN-γ signalling in primary human macrophages, with both the IFN-γ-induced STAT1 Ser727 and Tyr701 phosphorylation being impaired by PD98059 and SP600125 (Figure 4.7), These results therefore suggest that along with

the regulation of STAT1 Ser727 phosphorylation found in THP-1 cells, ERK and JNK may also regulate STAT1 activation in response to IFN-γ through phosphorylation at Tyr701 in primary human macrophages. Despite the different effect on Tyr701 phosphorylation, THP-1 and human primary macrophages exhibit a conserved action of ERK and JNK on the IFN-γ-stimulated phosphorylation at STAT1 Ser727, thereby adding support to the use of THP-1 cells as a useful cellular model system for investigating the roles of ERK and JNK in the regulation of STAT1 Ser727 phosphorylation in response to IFN-γ in human macrophages.

Table 4.4 Percentage of average suppression from three independent experiments of the IFN-γ-stimulated STAT1 phosphorylation by each inhibitor compared to DMSO treated control cells

Inhibitor (μM)	Targeted Kinase	Ser727	Tyr701
PD98059 (50)	ERK	80%	No change
U0126 (20)	ERK	38%	No change
SP600125 (30)	JNK	73%	No change
SB203580 (30)	P38	No change	No change
Apigenin (20)	CK2	40%	No change
LY294002 (30)	PI3K	33%	No change
Rottlerin (10)	PKC-δ	50%	65%
KN93 (20)	CaMK II	50%	26%

Absence of suppression of IFN-γ-stimulated STAT1 phosphorylation by pre-treatment of the cells with SB203580 (Figure 4.2C) indicates that p38 is not obligatory for the IFN-γ-mediated STAT1-dependent signalling. However, SB203580 at 10μM has been reported to induce JNK and ERK activities in human erythroleukaemia cell line TF-1((Birkenkamp et al., 2000). We cannot thus at

present rule out the possibility, that such increased ERK and JNK activities are also occurring in THP-1 macrophages and potentially masking the effect of SB203580 on STAT1 serine phosphorylation. Indeed, Kovarik et al. (2001) have shown that in mouse fibroblast cells, pre-treatment with SB203580 at 5μM further enhances the IFN-γ-stimulated STAT1 Ser727 phosphorylation possibly through increasing ERK and JNK activities. Moreover, Ramsauer et al. (2002) showed that p38 MAPK enhanced the IFN-γ-stimulated STAT1-dependent transcription independently of Ser727 phosphorylation. Therefore, although it appears that SB203580 failed to attenuate the IFN-γ-induced STAT1 phosphorylation, further experiments will be required to firmly exclude the possible role of p38 MAPK in the regulation of STAT1-dependent transcription in response to this cytokine in human macrophages.

As shown in Figure 4.10 and Table 4.5, transfection of the cells with DN forms of Ras, Raf-1, MEK1, ERK1, ERK2 and JNK resulted in a large decrease in the IFN-γ-induced luciferase expression regulated by tandem GAS elements (STAT1 consensus sites) in the 3xly6e vector, thereby confirming the crucial roles of the Ras—Raf-1—MEK—ERK1/2 pathway along with JNK in the IFN-γ-stimulated, STAT1-dependent transcriptional activity. Previous studies have demonstrated an important role for STAT1-binding GAS sites in the IFN-γ-mediated induction of MCP-1 gene expression (Valente et al., 1998; Zhou et al., 1998; Zhou et al., 2001). The inhibition observed of the IFN-γ-induced activation of MCP-1 promoter in the MCP[213]Luc plasmid by these DN mutants further supports a potentially important role for Ras—Raf-1—MEK—ERK along with the JNK pathway in the regulation of gene transcription downstream of STAT1 in response to this cytokine. It should however be noted that the inhibition of the IFN-γ-induced MCP[213]Luc activity by these DN mutants was much less extensive than that observed for 3xly6e. This suggests that an alternative, STAT1-independent signalling pathway might operate alongside a STAT1-dependent mechanism or in its absence, in the regulation of MCP-1 expression by IFN-γ. This assumption might explain the finding by Gil et al.

(2001) that MCP-1 expression is inducible by IFN-γ in both wild type and STAT1-deficient macrophages. The exact STAT1-independent mechanisms underlying the IFN-γ-induced MCP-1 expression are not fully understood. It is however worth noting that the increased expression of STAT3 in STAT1-deficient mouse embryonic fibroblasts has been shown to mediate the induction of SOCS-3 expression by IFN-γ (Ramana et al., 2005). More work will be needed to determine whether a similar STAT3-dependent mechanism operates to maintain the induction of MCP-1 expression by IFN-γ in the absence of STAT1.

Table 4.5 The average percentage inhibition of 3xly6e and MCP[213]Luc activity from three independent experiments by co-transfection of cells with each DN expression plasmid in the presence of IFN-γ

	Ras	Raf-1	MEK1	ERK1	ERK2	JNK
3xly6e	71	67	62	70	85	56
MCP[213]Luc	34	30	35	24	47	28

The role of ERK in the IFN-γ-mediated, STAT1-dependent gene expression was also confirmed by RT-PCR experiments. Pre-treatment of the cells with PD98059 inhibited the mRNA expression of MCP-1, MIP-1β, IP-10, ICAM-1 and SOCS-1 in a concentration-dependent manner (Figure 4.11). STAT1-dependent pathway has been shown to mediate the regulation of all these genes by IFN-γ (sections 3.1.5 and 4.1.4). Taken together with previous data on the effect of PD98059 on the IFN-γ-induced STAT1 Ser727 phosphorylation along with co-tranfection experiments using DN plasmids (Figures 4.2 and 4.10), the results suggest that the ERK pathway regulates STAT1-dependent gene expression in response to IFN-γ, at least in part, through the modulation of STAT1 phosphorylation at Ser727.

Gough et al. (2007) recently proposed a JAK1/STAT1-independent role for MEK/ERK in the regulation of IFN-γ response genes in mouse embryonic fibroblasts. It would be of interest to know whether this is also the case in human macrophages. As shown in Figure 4.11, pre-treatment of the cells with PD98059 failed to suppress the IFN-γ-induced ICERI/II expression, which has been shown to be potentially regulated through a JAK/STAT1-independent pathway (Harvey, 2006). Thus at least on the basis of the limited genes that we have studied in human macrophages, ERK appears to regulate the IFN-γ-mediated gene expression predominantly via its action on STAT1 activity. However, STAT1-independent pathways have also been shown to be involved in the regulation of MCP-1 and MIP-1β expression by IFN-γ (sections 3.1.5 and 4.1.4). We can therefore not rule out the possibility that ERK could regulate the expression of these two genes via a STAT1-independent mechanism alongside with its action on STAT1 activity.

The ERK and the JNK activity was shown to be induced by the addition of IFN-γ by about 3- and 1.5-fold respectively at the 30min time point (Figure 4.13), thereby further confirming the function of these two kinases in mediating IFN-γ signalling. The kinetics of ERK activity (Figure 4.14) in the presence of IFN-γ is consistent with the previous data presented in section 3.2.5, where the IFN-γ-mediated STAT1 Tyr701 and Ser727 phosphorylation reached its peak at 30-60min and 3h respectively. The kinase activity assays further support that ERK and JNK pathways may act upstream of STAT1 in mediating IFN-γ signalling in macrophages.

4.4.2 Roles of CK2 and PI3K in the IFN-γ-mediated induction of STAT1 phosphorylation

Pre-treatment of the cells with apigenin (CK2 inhibitor) at 20μM and LY294002 (PI3K inhibitor) at 30μM resulted in 40% and 33% inhibition, respectively, of the IFN-γ-induced STAT1 phosphorylation at Ser727, while the phosphorylation at

Tyr701 was unaffected (Figure 4.2 and Table 4.4). CK2 and PI3K might thus play an important role in the regulation of STAT1 activity specifically through the modulation of phosphorylation at Ser727 in response to IFN-γ. The results are consistent with previous findings in mouse macrophages (Harvey, 2006) and in mouse embryonic fibroblasts (Nguyen et al., 2001). The data are also in keeping with previous results on the effect of these two inhibitors on the IFN-γ-induced MCP-1 expression presented in Chapter 3 (Figures 3.5-3.8). Overall, CK2 and PI3K probably exert their roles in regulating IFN-γ-dependent gene expression in human macrophages via modulating STAT1 Ser727 phosphorylation.

As discussed earlier in Chapter 3 (section 3.3), although LY294002 at 50μM has been reported to inhibit the IL1-induced MCP-1 expression, through a PI3K-independent mechanism (Choi et al., 2004), work in our laboratory has previously confirmed the specific action of LY294002 at the concentrations used in this study (10-30μM) on the PI3K/Akt-dependent pathway (Harvey, 2006). Harvey (2007) has also demonstrated that a DN form of Akt, a key PI3K downstream effector, largely suppressed the IFN-γ-induced luciferase activity from 3xly6e and MCP[213]Luc plasmids. Therefore, the results presented in this study add further support to the potential role of the PI3K/Akt pathway in the regulation of IFN-γ-mediated MCP-1 expression along with STAT1 phosphorylation. To further confirm such role, siRNA for Akt available in our laboratory could facilitate our investigation via RNAi-mediated knock-down approaches.

4.4.3 Roles of PKC-δ and CaMK II in the IFN-γ-mediated induction of STAT1 phosphorylation

Inhibitors for PKC-δ (rottlerin) at 10μM and CaMK II (KN93) at 20μM attenuated the IFN-γ-induced STAT1 Ser727 phosphorylation by approximately 50% in both cases (Figure 4.4, Table 4.4). The results on PKC-δ are in line with previous findings in human acute promyelocytic leukemia cell line NB-4 (Deb et al., 2003)

and in the mouse macrophage cell line RAW264.7 (Kwon et al., 2007), while the observed results on CaMK II are consistent with previous studies in the mouse fibroblasts cell line NIH3T3 (Nair et al., 2002). However, although these three studies all demonstrated insensitivity of STAT1 Tyr701 phosphorylation to rottlerin and KN93, results in this thesis clearly show that these two inhibitors suppress STAT1 Tyr701 phosphorylation by 65% and 26% respectively (Table 4.4). It thus appears that in THP-1 macrophages, PKC-δ and CaMK II regulate IFN-γ-mediated signalling pathway through modulation of STAT1 phosphorylation at both Tyr701 and Ser727 sites. The discrepancy observed in the THP-1 cell line with the human promyelocytic leukemia cell line NB-4, mouse macrophage cell line RAW264.7 and the mouse fibroblast cell line NIH3T3 could probably reflect species- and cell type-specific action of the kinases, although additional experiments via different approaches will be required to confirm the role of these two kinases in THP-1 cells. Moreover, rottlerin has also been shown to prevent the IFN-γ-induced expression of all the marker genes analysed, including MCP-1, ICAM-1, SOCS-1 and ICERI/II in a dose-dependent manner. The results thereby suggest a potentially general role for PKC-δ in the IFN-γ-mediated induction of gene expression in human macrophages, via modulating the phosphorylation of STAT1 at Tyr701 and Ser727.

In the light of a recent report by Rybin (2008) that showed that PMA activates and phosphorylates PKC-δ at Tyr311, it is possible that in PMA-differentiated THP-1 cells used during the course of this study, PMA and IFN-γ could synergistically phosphorylate PKC-δ, which in turn stimulates the phosphorylation of STAT1 at Ser727 and Tyr701 sites. Inhibition of PKC-δ tyrosine phosphorylation by rottlerin (Soltoff, 2001) might result in the inhibition of STAT1 phosphorylation at both sites (Figure 4.20). Further approaches such as the use of DN mutants or siRNA against PKC-δ are required to further confirm the role of this kinase in IFN-γ signalling.

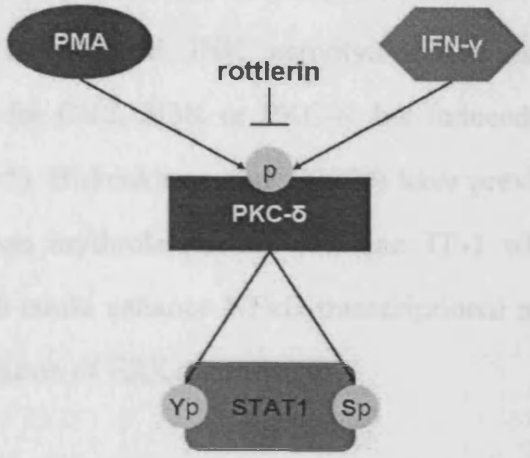


Figure 4.20 Potential synergistic actions of IFN-γ and PMA on PKC-δ activity and phosphorylation of its potential downstream effector STAT1

In the hypothetical pathway, PMA and IFN-γ synergistically induce tyrosine phosphorylation of PKC-δ, which could in turn phosphorylate STAT1 at Ser727 and Tyr701. Rottlerin, a specific inhibitor of PKC-δ, represses the tyrosine phosphorylation of the kinase. Abbreviations: PMA, phorbol 12-myristate 13-acetate; IFN, interferon; PKC, protein kinase C; STAT, signal transducers and activators of transcription; Y, Tyr701; S, Ser727; p, phosphorylation

4.4.4 Cross-talk between ERK, JNK and other kinases in IFN-γ-stimulated signalling pathways

A number of previous studies have demonstrated potential functional interactions between kinases acting upstream of STAT1 in response to IFN-γ. For example, Deb (2003) suggested a JAK—PI3K—PKC-δ pathway in stimulating STAT1 Ser727 phosphorylation in human acute promyelocytic leukemia cell line NB-4. Similarly, a PI3K—MAPK—STAT1 (Ser727) pathway has been demonstrated by Choudhury et al. (2004) in rat glomerular mesangial cells. Studies in this thesis on the effect of a number of kinase inhibitors on the ERK and JNK activities in THP-1 cells also suggest potential interactions (Figure 4.15). For example, whilst the IFN-γ-induced ERK activity was not attenuated following inhibition of JNK, CK2, PI3K or PKC-δ,

it was enhanced further by inhibitors of JNK, CK2 and PI3K. A similar result was obtained for the IFN-γ-induced JNK activity, which was not suppressed by inhibitors specifying for CK2, PI3K or PKC-δ, but induced by the ERK inhibitor PD98059 (Figure 4.15). Birkenkamp et al. (2000) have previously shown a similar phenomenon in human erythroleukaemia cell line TF-1 where inhibition of p38 MAPK by SB203580 could enhance NFκB transcriptional activity associated with increased phosphorylation of ERK1/2 and JNK.

One of the most likely explanations for such further enhancement of kinase activities is functional redundancy between signalling pathways, so that blocking one pathway might ultimately lead to increased activity of other routes to maintain a fully functional cellular response. As IFN-γ is a key player in the immune system, such redundancy in its signalling might represent an essential safety mechanism to ensure the maintenance of the whole immune system during the failure of one element (Thrane et al., 2007).

4.4.5 Roles of ERK and JNK in the IFN-γ-induced adhesion of monocytes to endothelial cells

Work presented in this chapter has shown significant roles of ERK and JNK pathways in mediating the IFN-γ-induced gene expression, many of which are largely involved in the adhesion of monocytes/macrophages to endothelial cells. It is therefore possible that the ERK and the JNK pathways also contribute to the IFN-γ-induced adhesion of monocytes to endothelial cells (Figure 4.17). Consistent with this notion, pre-treatment of the cells with U0216 (ERK inhibitor), SP600125 (JNK inhibitor) and combination of PD98059 (ERK inhibitor) and SP600125 attenuated the adhesion of U937 to HUVEC in response to the cytokine. PD98059 alone, however, did not inhibit, but instead, induced such adhesion (Figures 4.18 and 4.19). The exact underlying reason for the opposite effect of PD98059 and U0126 in such

adhesion is not clear at present, although we suspect that the differences in molecular mechanisms used by these two inhibitors might play a role. In addition, keeping with a previous study by Navarro et al. (2003), the PI3K pathway inhibitor, LY294002 was also shown to reverse the IFN-γ-stimulated adhesion of monocytes to endothelial cells.

It is worth noting that as addition of each inhibitor resulted in a low viability of HUVEC (Figures 4.20 and 4.21), we cannot rule out the possibility that the reduced adhesion may be due to decrease viability of HUVEC. Interestingly, although pre-treatment of HUVEC with PD98059 resulted in viability that was comparable to the other inhibitors, the adhesion was increased. It is therefore difficult to come to any firm conclusions on whether the inhibition of adhesion was due to the specific action of the inhibitors or because of reduced HUVEC viability. The low viability of HUVEC is probably due to the prolonged period of incubation (31h), and alternative approaches will thus be required in the future to confirm these results. For example, siRNA form of each kinase might provide valuable alternative to the use of pharmacological inhibitors. Our laboratory has recently adapted the adenovirus-mediated small hairpin RNA (shRNA) knock-down approach, which could be employed in the study of adhesion assay in the future.

CHAPTER FIVE:

EFFECTS OF THE THERAPEUTIC AGENTS ON IFN-γ SIGNALLING

Chapter 5: Effects of the therapeutic agents on IFN-γ signalling

5.1 Introduction

As detailed in the General Introduction (section 1.8), the NRs, PPARs and LXRs are recently emerging as crucial regulators of inflammation, and compelling evidence suggests that ligand-dependent activation of these NRs exert profound anti-inflammatory and protective effects in atherosclerotic development [see (Repa and Mangelsdorf, 2002; Barish, 2006; Bensinger and Tontonoz, 2008; Nomiyama and Bruemmer, 2008) for reviews]. Although statins are prescribed as cholesterol-lowering drugs, animal studies and clinical trails have recently confirmed that in addition to the cholesterol-lowering effects, statins also exert their athero-protective roles via cholesterol-independent effects, so called 'pleiotropic effects'. These effects are largely dependent on their anti-inflammatory and immunosuppressive actions [see (Mach, 2002b, 2003; Kronmann et al., 2007) for reviews].

As detailed in the General Introduction (section 1.5), IFN-γ is a key pro-inflammatory cytokine regulating atherosclerotic development, and a large number of studies have suggested that these therapeutic agents target inflammation by repressing the IFN-γ-mediated expression of inflammatory response genes in macrophages [see (Straus and Glass, 2007; Rigamonti et al., 2008) for reviews]. To date, the molecular mechanisms underlying the anti-inflammatory actions of PPAR and LXR ligands along with statins remain largely unclear, particularly in relation to the IFN-γ-dependent signalling and the JAK-STAT pathway. Therefore, investigating the molecular basis of the anti-inflammatory actions of these therapeutic agents in the IFN-γ-mediated signalling and the JAK-STAT pathway could potentially lead to the

identification of novel targets for therapeutic intervention of atherosclerosis. Work in this chapter was carried out with the aim of analysing the potential effects of PPARs and LXRs ligands along with statins on the IFN-γ-regulated gene expression and their impact on the IFN-γ-mediated signalling and cellular responses in human macrophages.

5.1.1 Experimental strategy

PMA-differentiated THP-1 macrophages were employed again as discussed in previous chapters. To initiate the investigation, two types of statins (lovastatin and simvastatin) and synthetic agonists for PPAR-γ (BRL49653), -α (GW7647) and LXRs (TO901317) were initially employed to screen for their effects on the IFN-γ-mediated expression of a range of inflammatory genes. Lovastatin and simvastatin were chosen here because they are two of the most commonly used statins for treating hypercholesterolemia and have both been employed for investigating the effect of statins on IFN-γ-mediated inflammatory gene expression in macrophages (Mach, 2002a; Veillard et al., 2006; Lee et al., 2007b; Lee et al., 2008). Anti-inflammatory effect of both statins *in vivo* has also been confirmed in animal studies (Chauhan et al., 2004; Ulivieri et al., 2008; Winkler et al., 2009). Anti-inflammatory and athero-protective roles of PPARα ligands, such as GW7647 and PPARγ ligands, such as BRL49653 (also called 'rosiglitazone') have been consistently confirmed in mouse models of atherosclerosis (Collins et al., 2001; Chen et al., 2001b; Duez et al., 2002; Zuckerman et al., 2002; Li et al., 2004) (see Table 1.6 for a list of animal studies). On the other hand, contradictory results for the effect of PPARδ ligands in atherosclerotic lesion development have been reported. Li et al. (2004) showed that the PPARδ ligand GW0742 had no effect on atherosclerotic lesion size in the LDLR-deficient male mice. In contrast, the same ligand has been reported to reduce the lesion size in LDLR-/- female mice (Graham et al., 2005). Although the exact reason for these contrary findings remains unclear at present, the gender of the mice might have played an

important role. Due to the potential complexity of the mechanisms underlying the PPARδ ligands, we decided to initiate our investigation on the potential roles of PPARs on IFN-γ responses with only PPAR-α and -γ, excluding PPARδ in the work presented in this study. TO901317 is a commonly used synthetic agonist for the LXRs and has been widely used to investigate the anti-inflammatory and anti-atherogenic effects of LXRs activation in animal models (Dai et al., 2007; Ou et al., 2008; Peng et al., 2008). In addition, TO901317 has been routinely used for studies on THP-1 macrophage in our laboratory (Huwait, 2008).

Following screening for the effects of these compounds on the IFN-γ-induced gene expression, their potential impact on IFN-γ-dependent signalling was also confirmed by analysing their effects on the IFN-γ-induced STAT1 phosphorylation at Ser727 and Tyr701. In addition to the synthetic agonists for PPARs and LXRs as discussed above, endogenous ligands for PPARγ and LXRs were also included. 15d-PGJ2 is a widely used naturally occurring PPARγ ligand and several studies have demonstrated that it shows greater transrepression activity on inflammatory gene expression than the various types of synthetic PPARγ agonists (Jiang et al., 1998; Ricote et al., 1998; Chawla et al., 2001). In the case of the LXRs, the endogenous ligand 22(R)-hydroxycholesterol (22R) was selected as it is one of the most commonly used ligand for activating these NRs (Joseph et al., 2003; Piraino et al., 2006), and has been routinely used with studies on THP-1 macrophages in our laboratory (Greenow, 2004; Huwait, 2008). In addition, LXRs are known to form functional heterodimers with RXRs to bind to the LXRE in the promoter of target genes and activate their transcription (Rizzo and Fiorucci, 2006). It has been shown that LXR/RXR can be activated by ligands for both LXR and RXR, either separately or in synergy (Aranda and Pascual, 2001). It was thus decided to also include the agonist for RXRs (9-*cis*-retinoic acid [9cRA]) and analyse the individual and combinatorial effect of 22R and 9cRA on the IFN-γ-induced STAT1 phosphorylation.

The effect of statins and agonists for PPARγ and LXRs on the IFN-γ-mediated gene expression and the activation of STAT1 response element were again confirmed using DNA constructs MCP[213]Luc and 3xly6e in transfection experiments, as discussed in Chapter 4. Finally, the studies were extended to the investigation of the effect of PPAR and LXR ligands on the IFN-γ-mediated changes in a key cellular response relevant to atherosclerosis, the adhesion of monocytes to endothelial cells, as discussed in Chapter 4 (see Figure 5.1 for the overall strategy).

It is worth noting that in order to prevent the potential interference by fatty acids and other lipids that might exist in FCS, which could possibly activate PPARs and LXRs, delipidated FCS was used in experiments employing these NRs. Numerous laboratories have employed delipidated serum for such studies (Costet et al., 2003; Quinn et al., 2005; Beyea et al., 2007). Serum delipidation has been routinely carried out in our laboratory and not been found to show any deleterious effect on cellular viability and cytokine responses (Huwait, 2008).

5.1.2 Selection of genes for study

In order to analyse the potential effect of statins and agonsits for PPARs and LXR in the IFN-γ-induced gene expression, the genes employed in previous chapters, including MCP-1, IP-10, MIP-1β, ICAM-1, SOCS-1 and ICER, were selected here again for further investigation. In addition, the major histocompatibility complex (MHC) class II activator (CIITA) was included. MHC class II molecules are directly involved in the activation of T lymphocytes and in the control of the immune response. Most of the cells do not express MHC-II constitutively and become MHC-II positive upon activation by IFN-γ (Mach et al., 1996). This complex regulation is under the control by CIITA (Steimle et al., 1994). Aberrant class II MHC expression is associated with inflammatory diseases, including rheumatoid arthritis and multiple sclerosis (Mach et al., 1996).

The expression of CIITA is cell type-specific and tightly controlled by four independent promoters in humans (pI, pII, pIII and pIV). CIITA type I isoform is specifically and constitutively expressed in dendritic cells. By contrast, CIITA type III is mainly expressed by B lymphocytes and can be induced by IFN-γ in fibroblasts, endothelial cells and B lymphocytes. CIITA type IV is the major IFN-γ-inducible isoform expressed by a variety of cell types (Buttice et al., 2006). pIV has several important *cis*-acting elements including GAS, E-box and IRE. The GAS and E-box elements are bound cooperatively by activated STAT-1α and upstream regulatory factor-1 (USF-1) respectively. Activated STAT-1α also induces the expression of IRF-1, which then binds IRE in pIV (Piskurich et al., 2006). Histone acetylation at the CIITA pIV is increased following IFN-γ stimulation (Lee et al., 2008). Brg-1, which is needed to mediate chromatin remodelling, is also functionally required for CIITA gene transcription (Ni et al., 2005). In addition to pIV, other CIITA promoters can also be used to direct IFN-γ-inducible CIITA transcription in macrophages, such as pI (LeibundGut-Landmann et al., 2004). A number of studies have shown that statins inhibit IFN-γ-induced CIITA expression (Kwak et al., 2000; Sadeghi et al., 2001; Mach, 2002a; Youssef et al., 2002; Lee et al., 2008). In particular, Youssef (2002) showed that statins are not selective for pIV, but inhibit IFN-γ-inducible CIITA transcription in general. Therefore, CIITA pIII and pIV genes were selected here for work detailed in this chapter.

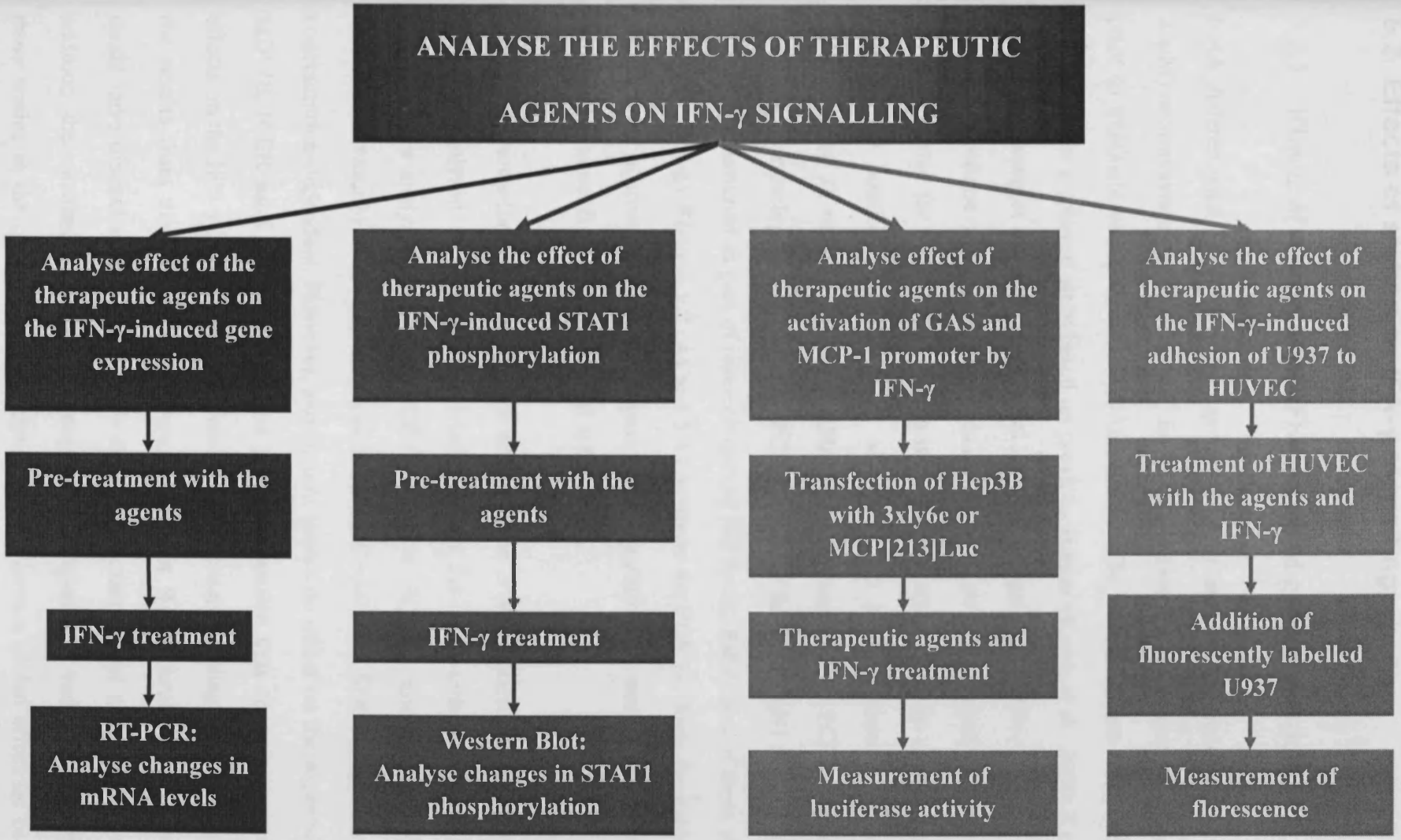


Figure 5.1 Experimental Strategy

The aim of the experimental strategy was to analyse the effect of a variety of potential therapeutic agents, including statins, agonists of PPAR-α and -γ along with LXRs on the IFN-γ-mediated signalling pathways. This was achieved by the analysis of the effects of these agents on the IFN-γ-induced gene expression, STAT1 phosphorylation and transactivation, MCP-1 promoter activation and monocyte-endothelial adhesion.

5.2 Effects of statins on IFN-γ signalling

5.2.1 Effects of statins on the IFN-γ-induced gene expression

PMA-differentiated THP-1 macrophages were pre-treated with lovastatin (5, 10 and 20μM) or simvastatin (1, 5 and 10μM) for 1h as indicated below (Figures 5.2 and 5.3) prior to treatment with IFN-γ (1000U/ml) for 3h. The concentrations of simvastatin and lovastatin employed were based on previous studies (Kwak et al., 2000; Kwak et al., 2001; Chung et al., 2002; Luan et al., 2003; Bruegel et al., 2006; Veillard et al., 2006). The volume of DMSO, which was added to the cells as a negative control, was the same as that for the highest concentration of the respective statin used in order to rule out the possibility of the effect of DMSO on gene expression. Following incubation for 3h with the cytokine, RNA was extracted and RT-PCR analysis was carried out for each gene. The optimal PCR conditions for CIITA pIII and pIV (Table 2.4) were determined as part of research carried out during the course of these studies (data not shown). Figures 5.2 (A) and 5.3 (A) show the PCR products for each gene resolved by agarose gel electrophoresis. Densitometric and statistical analysis is presented in panel B of Figures 5.2 and 5.3.

Figure 5.2 shows that lovastatin has no effect on the IFN-γ-induced expression of all the genes analysed. In contrast, simvastatin inhibits the IFN-γ-induced expression of several genes analysed, including MCP-1, ICAM-1, SOCS-1 and CIITA pIV. The effect of simvastatin on the expression of MCP-1, ICAM-1 and SOCS-1 appears to be concentration-dependent. However, simvastatin shows no effect on the expression of MIP-1β, ICER sand CIITA pIII (Figure 5.3). Simvastatin thus shows gene-specific effects on the IFN-γ-induced gene expression in human macrophages. Taken together, the results from these RT-PCR experiments suggest that different types of statins might have distinct actions on the IFN-γ-regulated macrophage gene expression. In addition, the constant expression of control genes (Figures 5.2 and 5.3) suggests that these statins at the concentrations analysed do not have a global effect on cellular

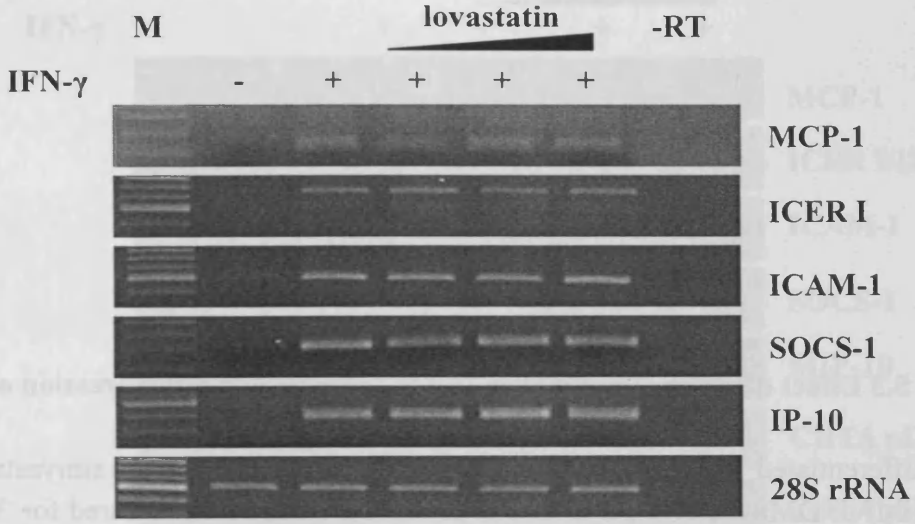
gene transcription.

In experiments described in this chapter involving the use of therapeutic agents on THP-1 macrophages, the viability of the cells was analysed by the trypan blue exclusion assay. All the results showed a negligible proportion of dead cells (< 10%) following treatment with IFN-γ in combination with each of the agent at the highest concentration employed (Table 5.1), indicating that the changes in mRNA or protein expression, if any, were not due to cell death, but because of the specific action of the relevant agent.

Figure 5.2 Effect of lovastatin on the IFN-γ-induced mRNA expression of genes

PMA-differentiated THP-1 macrophages were pre-treated for 1h with lovastatin (5, 10 and 20μM) or DMSO as a vehicle control. The cells were then cultured for 3h in the presence or the absence of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1, ICER, ICAM-1, SOCS-1, IP-10 and 28S rRNA. The amplification products were analysed by agarose gel electrophoresis and the images shown are representative of three independent experiments. M denotes the 100bp DNA ladder (Appendix I); –RT indicates a reaction in which no reverse transcriptase was used during the cDNA synthesis step (RNA was from cells incubated with DMSO and IFN-γ). Panel B shows the relative expression (mean ±SD) of the indicated gene normalised to the expression of 28S rRNA as determined by densitometric analysis from three independent experimental series (IFN-γ-treated, DMSO control assigned as 1). Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with lovastatin and IFN-γ (N/S, not significant).

(A)


M
lovastatin
-RT
IFN-γ
- + + + +
MCP-1
ICER I
ICAM-1
SOCS-1
IP-10
28S rRNA

(B)

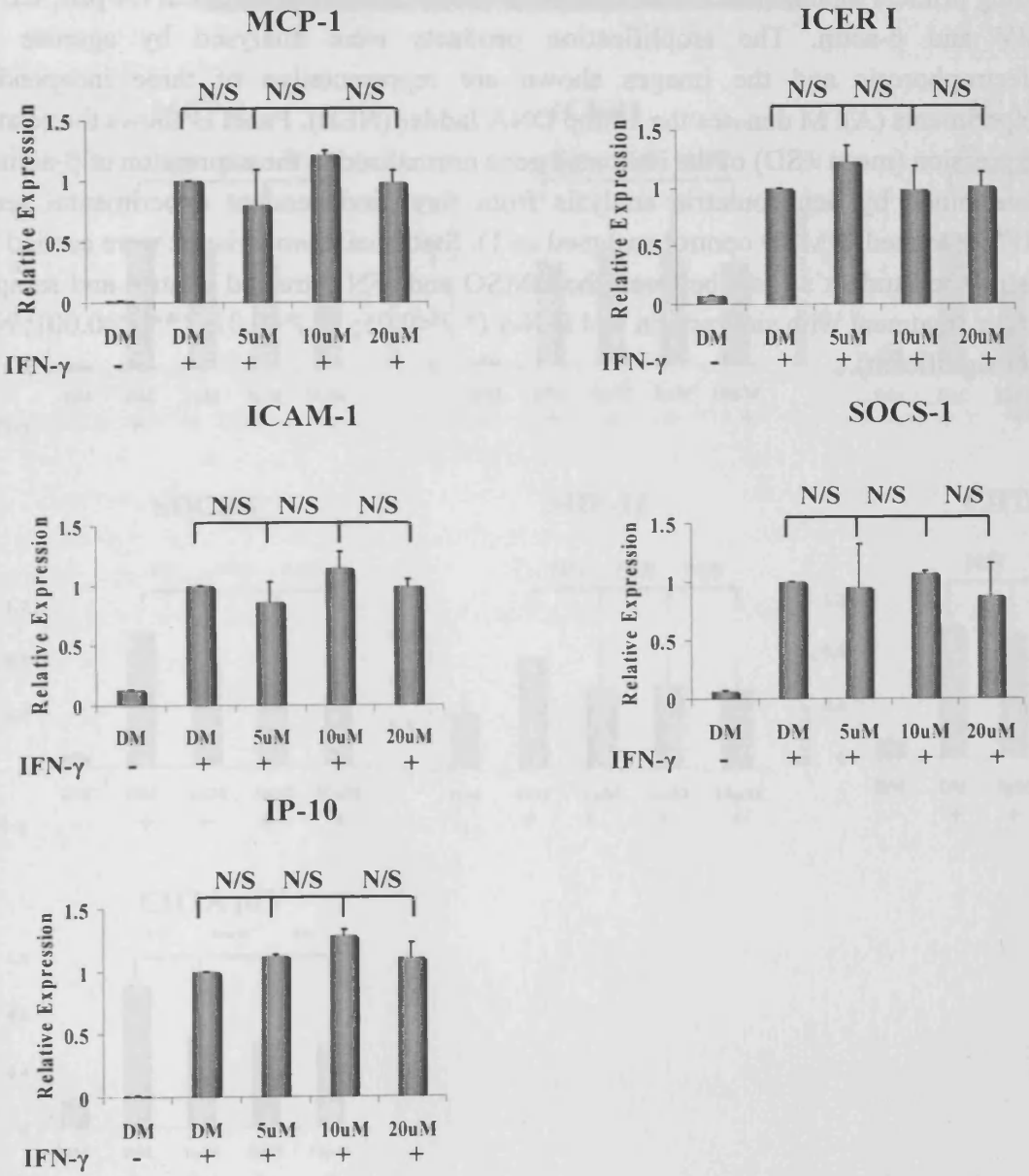
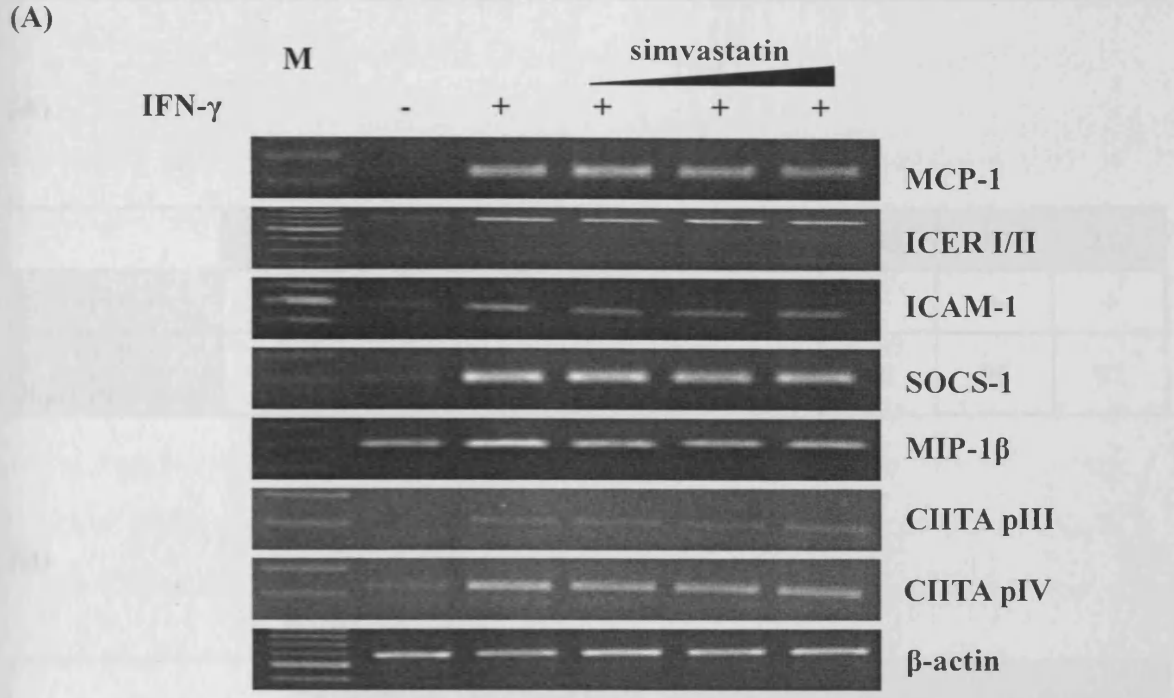
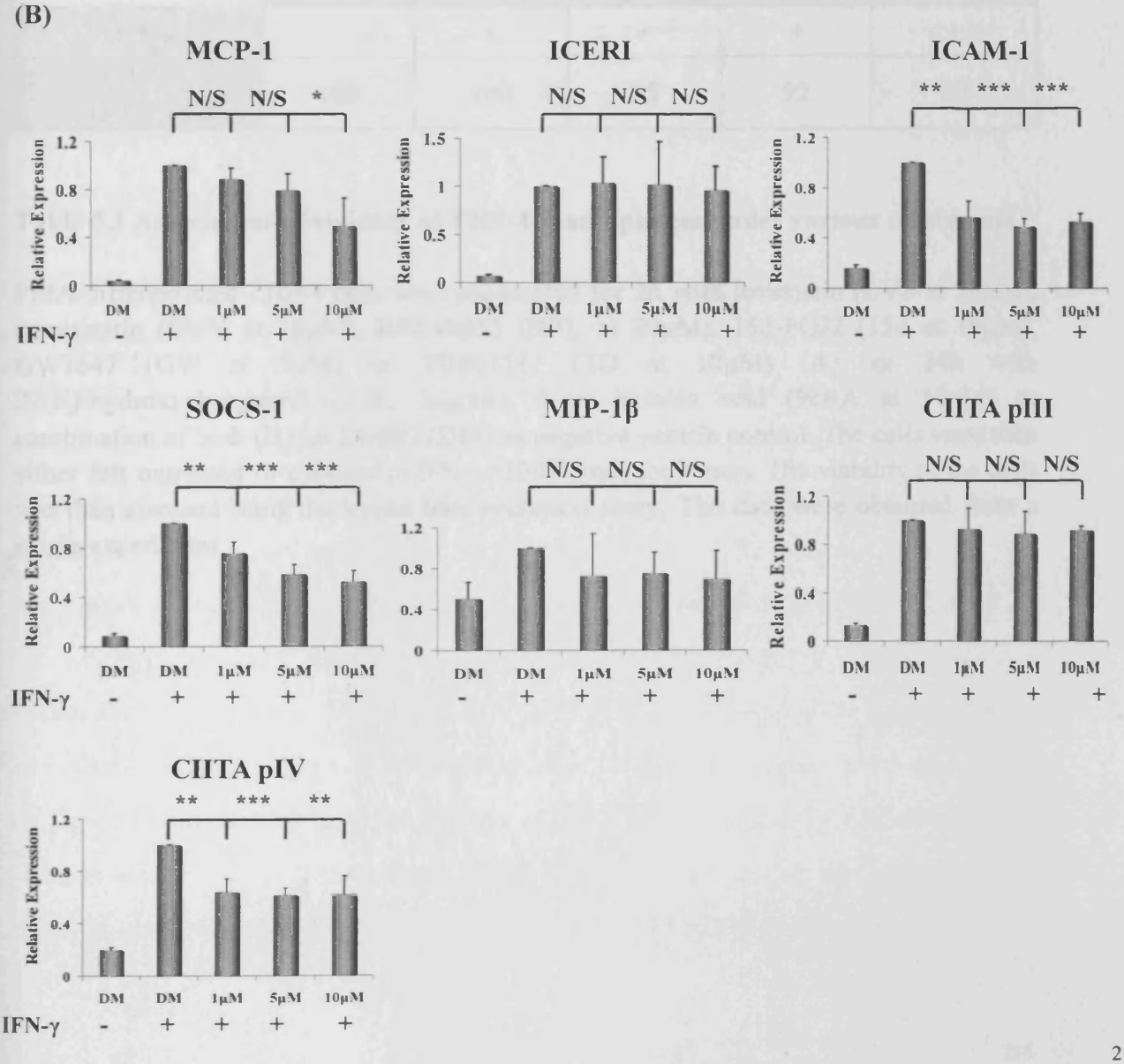

MCP-1
ICER I
ICAM-1
SOCS-1
IP-10
N/S N/S N/S
Relative Expression
1.5
1
0.5
0
DM DM 5uM 10uM 20uM
IFN-γ - + + + +

Figure 5.3 Effect of simvastatin on the IFN-γ-induced mRNA expression of genes

PMA-differentiated THP-1 macrophages were pre-treated for 1h with simvastatin (1, 5 and 10μM) or DMSO as a vehicle control. The cells were then cultured for 3h in the presence or the absence of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1, ICER, ICAM-1, SOCS-1, MIP-1β, CIITA pIII, CIITA pIV and β-actin. The amplification products were analysed by agarose gel electrophoresis and the images shown are representative of three independent experiments (A). M denotes the 100bp DNA ladder (NEB). Panel B shows the relative expression (mean ±SD) of the indicated gene normalised to the expression of β-actin as determined by densitometric analysis from three independent experimental series (IFN-γ-treated, DMSO control assigned as 1). Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with simvastatin and IFN-γ (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; N/S, not significant).


(A)
M
simvastatin
IFN-γ - + + + +
MCP-1
ICER I/II
ICAM-1
SOCS-1
MIP-1β
CIITA pIII
CIITA pIV
β-actin


(B)
MCP-1
N/S N/S *
ICERI
N/S N/S N/S
ICAM-1
** *** ***
SOCS-1
** *** ***
MIP-1β
N/S N/S N/S
CIITA pIII
N/S N/S N/S
CIITA pIV
** *** **
Relative Expression
DM DM 1μM 5μM 10μM
IFN-γ - + + + +

(A)

	DM	DM	LVS	SMS	BRL	15d	GW	TO
IFN-γ	-	+	+	+	+	+	+	+
THP-1 viability (%)	100	100	98	99	96	90	96	97

(B)

	DM	DM	22R	9cRA	22R+9cRA
IFN-γ	-	+	+	+	+
THP-1 viability (%)	100	100	95	92	90

Table 5.1 Assessment of viability of THP-1 macrophages under various treatments

PMA-differentiated THP-1 cells were pre-treated for 2h with lovastatin (LVS at 20μM), simvastatin (SMV at 10μM), BRL49653 (BRL at 20μM), 15d-PGJ2 (15d at 10μM), GW7647 (GW at 5μM) or TO901317 (TO at 10μM) (A), or 24h with 22(R)-hydroxycholesterol (22R, 2μg/ml), 9-*cis* retinoic acid (9cRA at 10μM) or combination of both (B), or DMSO (DM) as negative vehicle control. The cells were then either left untreated or exposed to IFN-γ (1000U/ml) for 30min. The viability of the cells was than assessed using the trypan blue exclusion assay. The data were obtained from a single experiment.

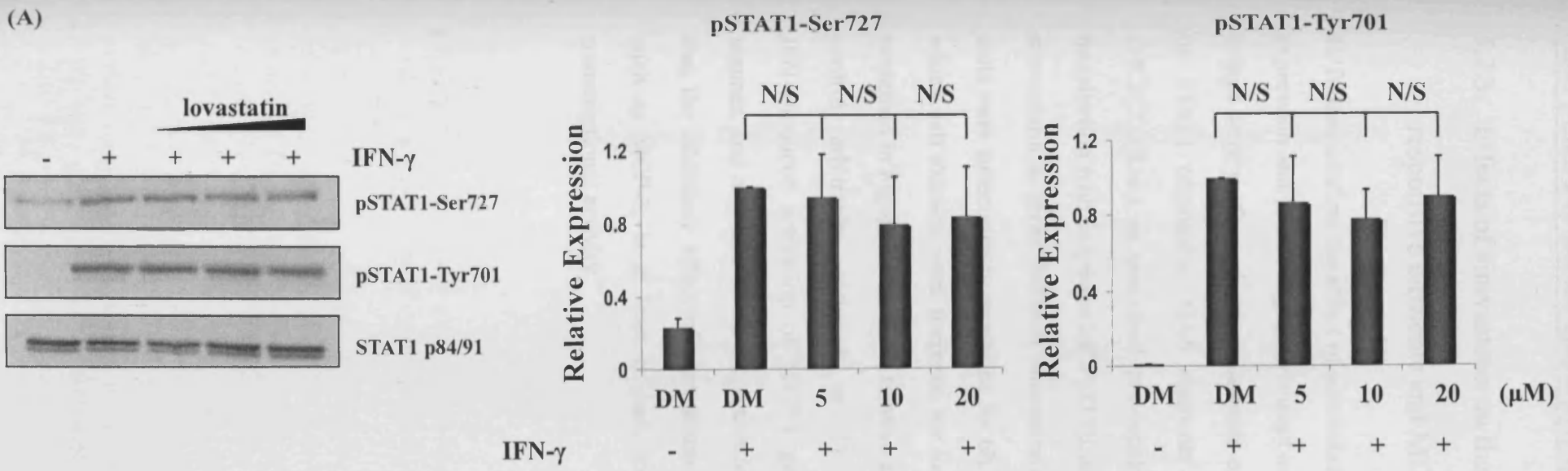
5.2.2 Effects of statins on the IFN-γ-induced STAT1 phosphorylation

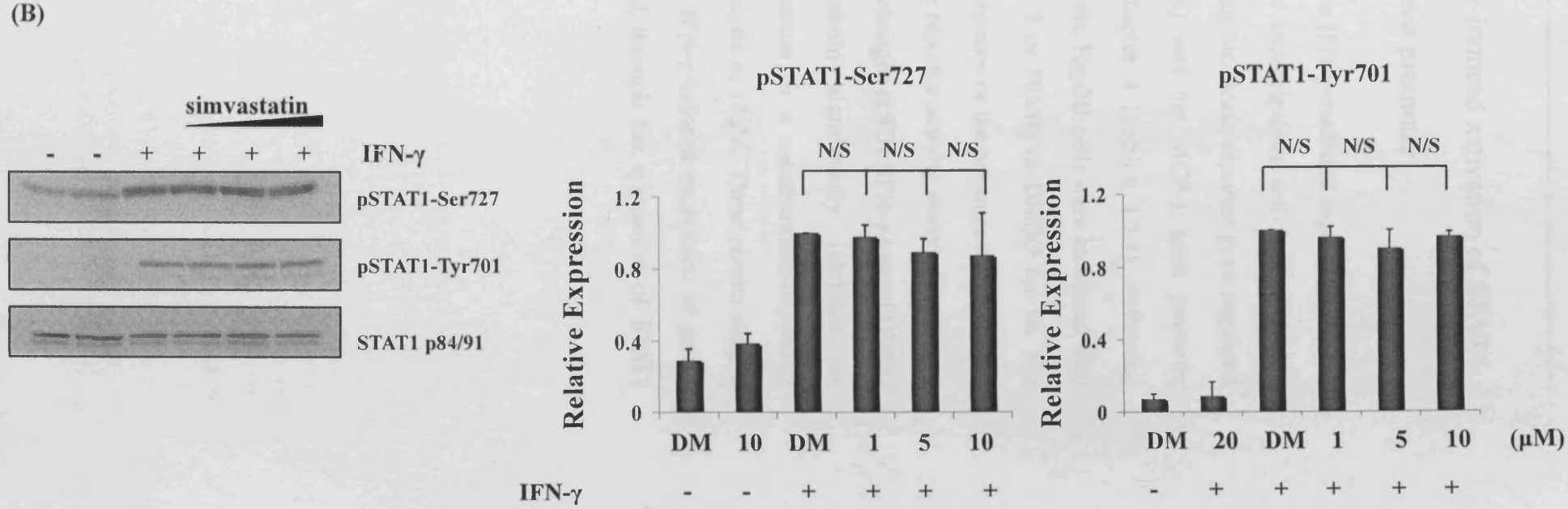
Lovastatin and simvastatin at a series of concentrations were employed again to investigate whether statins exert their effects on IFN-γ signalling through modulation of STAT1 phosphorylation. PMA-differentiated THP-1 macrophages were pre-treated with the respective statin for 1h prior to stimulation with IFN-γ (1000U/ml) for 30min. The volume of DMSO added to the cells as a vehicle control was the same as that for the highest concentration of each statin used in order to rule out the possibility of the effect of DMSO on STAT1 phosphorylation. Following incubation with the cytokine, whole cell proteins were extracted and western blot analysis was carried out as described before in Chapter 4. Densitometric analysis of the data was carried out and presented in Figure 5.4 Band intensity for phospho-STAT1 Ser727 and Tyr701 were normalised to total STAT1 (p84/91). Results are presented as the IFN-γ-induced expression levels in the presence of statins (±SD) normalised to the induction levels stimulated by the cytokine, which was arbitrarily assigned as 1. Student's *t* test was used to assess for statistical significance.

As shown in Figure 5.4 (A), lovastatin showed no statistically significant effect on the IFN-γ-induced STAT1 phosphorylation at either Ser727 or Tyr701. This is consistent with previous data from RT-PCR experiments which show that the IFN-γ-induced expression of marker genes analysed was not affected by lovastatin. These results together suggest that lovastatin probably does not target IFN-γ signalling, at least at the level of genes analysed in this study and the JAK-STAT pathway in THP-1 macrophages. Figure 5.4 (B) shows that simvastatin did not affect the IFN-γ-induced STAT1 phosphorylation. Having shown that simvastatin inhibits the IFN-γ-induced expression of some of the genes analysed (Figure 5.3), the results thereby suggest that simvastatin might affect the IFN-γ-induced expression of these genes independently of STAT1 phosphorylation. Moreover, the addition of simvastatin did not affect the phospho-STAT1 status in the absence of IFN-γ, thereby suggesting that simvastatin does not target the constitutive phosphorylation of STAT1 in THP-1 macrophages.

Figure 5.4 Effect of lovastatin and simvastatin on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 macrophages were pre-treated for 1h with lovastatin (A) and simvastatin (B) at three different concentrations as indicated or DMSO (DM, equal volume as the highest concentration of the respective statins) as a vehicle control. The cells were then cultured for 30min in the presence or the absence of IFN-γ. Cell protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with each type of statin versus IFN-γ-treated, DMSO control sample (N/S, not significant).


(A)
lovastatin
- + + + + IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
pSTAT1-Tyr701
N/S N/S N/S
Relative Expression
1.2
0.8
0.4
0
DM DM 5 10 20 (μM)
IFN-γ - + + + +


(B)
simvastatin
- - + + + + IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
pSTAT1-Tyr701
N/S N/S N/S
Relative Expression
1.2
0.8
0.4
0
DM 10 DM 1 5 10 (μM)
DM 20 DM 1 5 10 (μM)
IFN-γ - - + + + +
- + + + + +

5.2.3 Effects of simvastatin on the IFN-γ-induced activation of STAT1 responsive elements and MCP-1 gene promoter

To further confirm the effect of simvastatin on the IFN-γ-mediated regulation of gene expression and to investigate its impact on STAT1 transcriptional activity, transfection assays were performed using plasmids containing luciferase reporter gene regulated by STAT1 responsive, GAS elements (3xly6e) and the MCP-1 gene promoter (MCP[213]Luc) as described previously in Chapter 4 (section 4.3.4). Following transfection with 3xly6e or MCP[213]Luc plasmids, Hep3B cells were incubated with simvastatin at three different concentrations (1, 5 or 10μM) or DMSO for 1h. The cells were subsequently incubated for 6h in the absence or the presence of IFN-γ after which cell extracts were prepared for luciferase reporter activity assays. Results are presented in Figure 5.5 as mean relative activity changes (±SD) (IFN-γ-treated DMSO control arbitrarily assigned as 1). Simvastatin significantly inhibited the IFN-γ-induced activation of MCP-1 gene promoter in a concentration-dependent manner, and also attenuated the activation of 3xly6e at 10μM. These results suggest that the inhibitory effect of simvastatin on the IFN-γ-induced expression of genes, such as MCP-1, is at least in part, modulated through the regulation of STAT1 transcriptional activity.

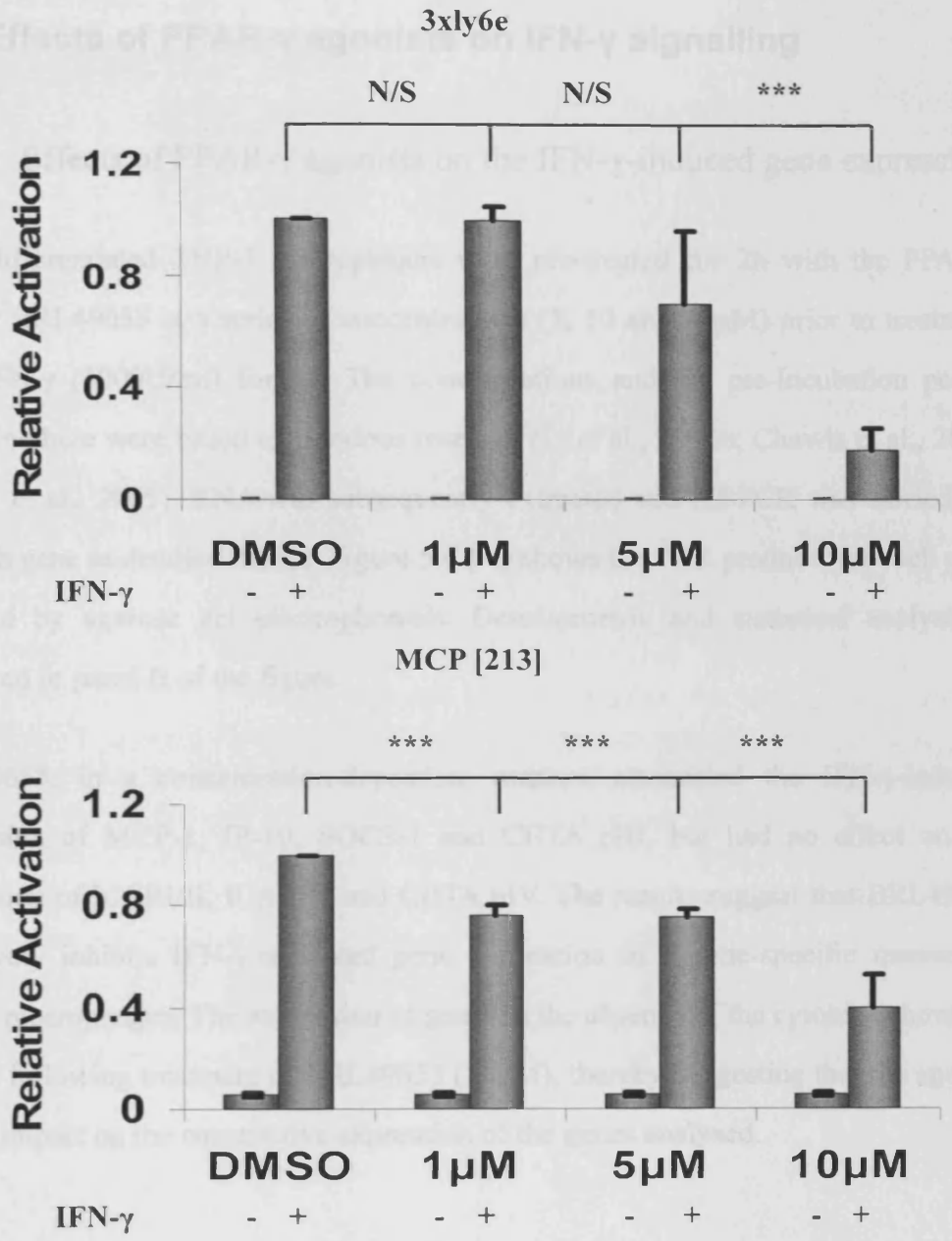


Figure 5.5 Effect of simvastatin on the stimulation of STAT1 responsive elements and the MCP-1 gene promoter activity by IFN-γ

Hep3B cells were transfected with plasmids 3xly6e or MCP[213]Luc. Following 1h incubation with simvastatin (1, 5 and 10μM) or DMSO, transfected cells were incubated for 6h in the absence or the presence of IFN-γ after which cell extracts were prepared for luciferase reporter activity assay. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated samples, from three independent experiments. The data was further normalised to the relative activation of IFN-γ-treated DMSO control sample (arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with simvasatin versus IFN-γ-stimulated DMSO control sample (***, $P<0.001$; N/S, not significant).

5.3 Effects of PPAR-γ agonists on IFN-γ signalling

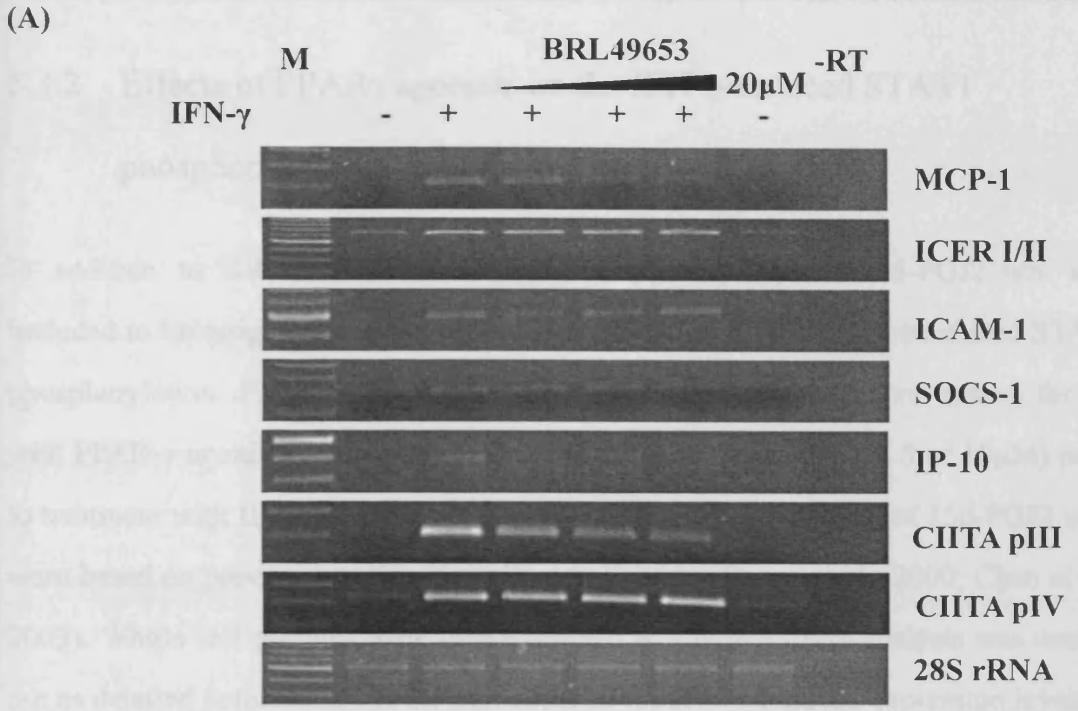
5.3.1 Effects of PPAR-γ agonists on the IFN-γ-induced gene expression

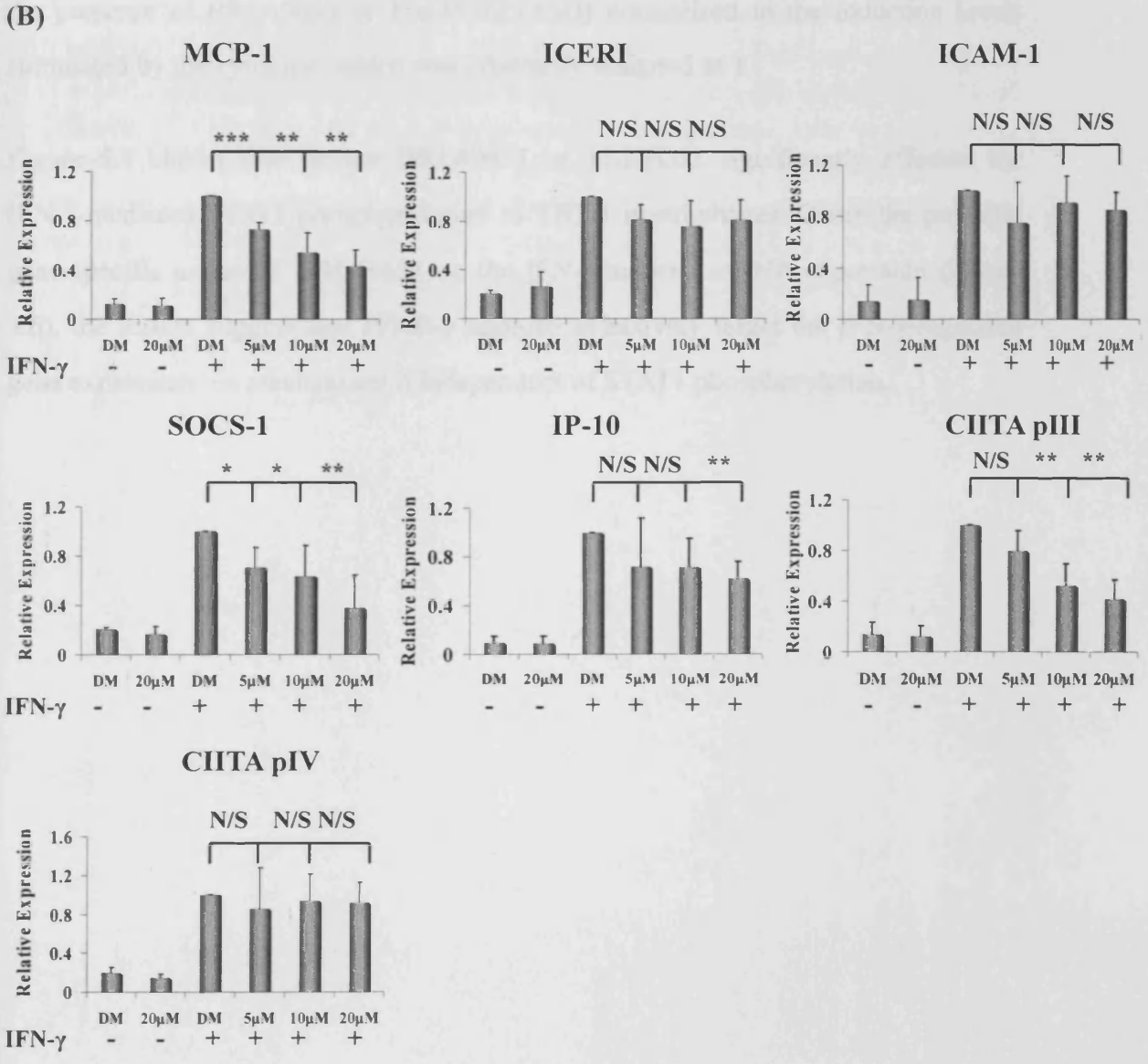
PMA-differentiated THP-1 macrophages were pre-treated for 2h with the PPAR-γ agonist BRL49653 at a series of concentrations (5, 10 and 20μM) prior to treatment with IFN-γ (1000U/ml) for 3h. The concentrations and the pre-incubation period employed here were based on previous research (Li et al., 2000b; Chawla et al., 2001; Crosby et al., 2005). RNA was subsequently extracted and RT-PCR was carried out for each gene as detailed before. Figure 5.6 (A) shows the PCR products for each gene resolved by agarose gel electrophoresis. Densitometric and statistical analysis is presented in panel B of the figure.

BRL49653, in a concentration-dependent manner, attenuated the IFN-γ-induced expression of MCP-1, IP-10, SOCS-1 and CIITA pIII, but had no effect on the expression of ICERI/II, ICAM-1 and CIITA pIV. The results suggest that BRL49653 selectively inhibits IFN-γ-regulated gene expression in a gene-specific manner in THP-1 macrophages. The expression of genes in the absence of the cytokine shows no change following treatment of BRL49653 (20μM), thereby suggesting that the agonist has no impact on the constitutive expression of the genes analysed.

Figure 5.6 Effect of BRL49653 on the IFN-γ-induced mRNA expression of genes

PMA-differentiated THP-1 macrophages were pre-treated for 2h with BRL49653 (5, 10 and 20μM) or DMSO as a vehicle control. The cells were then cultured for 3h in the presence or the absence of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1, ICER, ICAM-1, SOCS-1, IP-10, CIITA pIII, CIITA pIV and 28S rRNA. The amplification products were analysed by agarose gel electrophoresis and the images shown are representative of three independent experiments (A). M denotes the 100bp DNA ladder (Appendix I) and –RT indicates a reaction in which no reverse transcriptase was added during the cDNA synthesis step (RNA was from cells incubated with DMSO and IFN-γ). Panel B shows the relative expression (mean ±SD) of the indicated gene normalised to the expression of 28S rRNA as determined by densitometric analysis from three independent experimental series (IFN-γ-treated, DMSO control assigned as 1). Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with BRL49653 and IFN-γ (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; N/S, not significant).


(A)
M
BRL49653
20μM
-RT
IFN-γ - + + + + -
MCP-1
ICER I/II
ICAM-1
SOCS-1
IP-10
CIITA pIII
CIITA pIV
28S rRNA


(B)
MCP-1
ICERI
ICAM-1
SOCS-1
IP-10
CIITA pIII
CIITA pIV
Relative Expression
DM 20μM DM 5μM 10μM 20μM
IFN-γ - - + + + +
*** ** **
N/S N/S N/S
N/S N/S N/S
* * **
N/S N/S **
N/S ** **
N/S N/S N/S

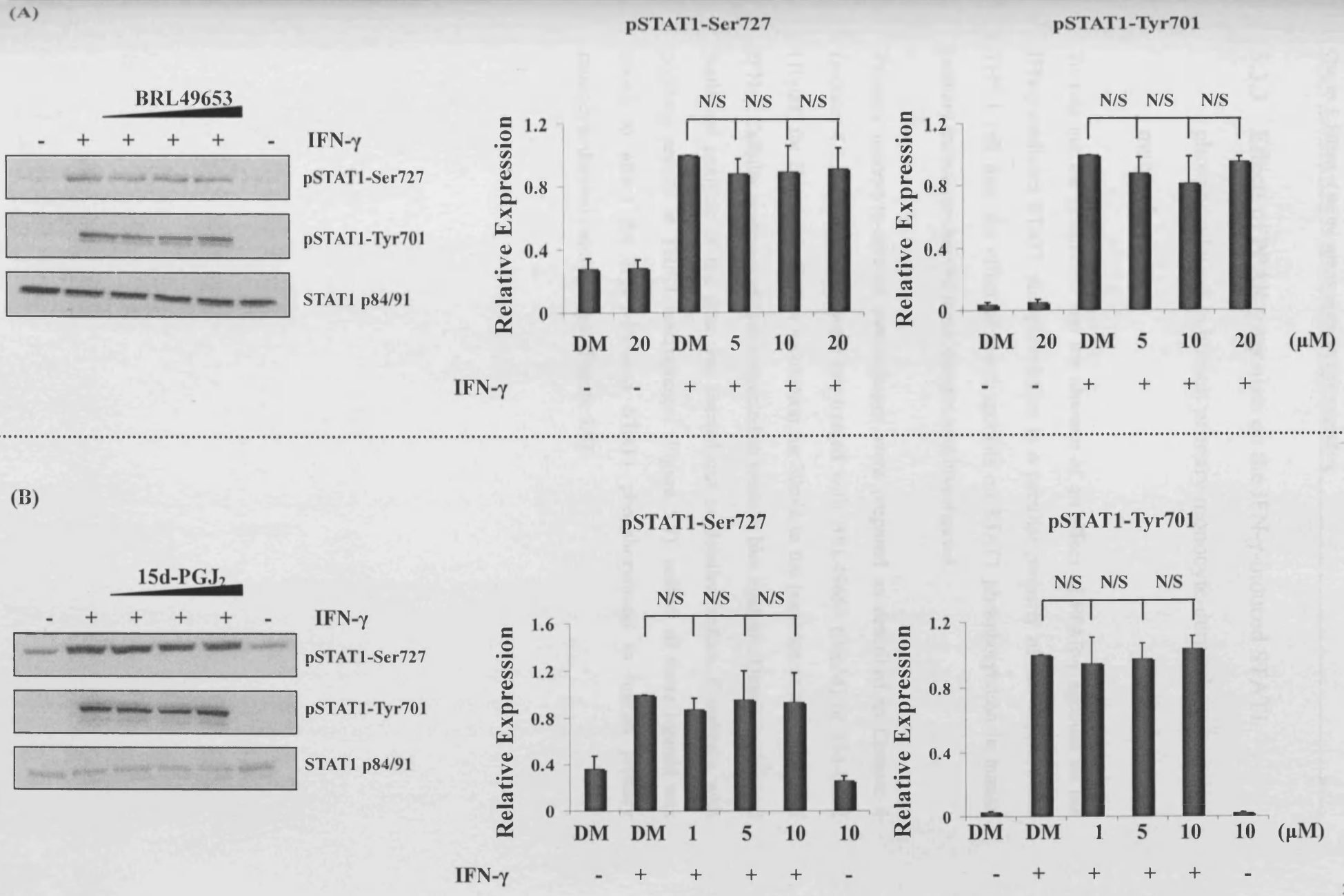
5.3.2 Effects of PPARγ agonists on the IFN-γ-induced STAT1 phosphorylation

In addition to BRL49653, the endogenous PPAR-γ ligand, 15d-PGJ2 was also included to investigate the effect of PPAR-γ activation on the IFN-γ-mediated STAT1 phosphorylation. PMA-differentiated THP-1 macrophages were pre-treated for 2h with PPAR-γ agonists BRL49653 (5, 10 or 20μM) or 15d-PGJ2 (1, 5 or 10μM) prior to treatment with IFN-γ (1000U/ml) for 30min. The concentrations of 15d-PGJ2 used were based on previous studies (Castrillo et al., 2000; Straus et al., 2000; Chen et al., 2003). Whole cell proteins were then extracted and western blot analysis was carried out as detailed before. Results are presented as the IFN-γ-induced expression levels in the presence of BRL49653 or 15d-PGJ2 (±SD) normalized to the induction levels stimulated by the cytokine, which was arbitrarily assigned as 1.

Figure 5.7 shows that neither BRL49653 or 15d-PGJ2 significantly affected the IFN-γ-mediated STAT1 phosphorylation in THP-1 macrophages. Given the potential gene-specific action of BRL49653 on the IFN-γ-induced mRNA expression (Figure 5.6), the results suggest that PPAR-γ agonists selectively target the IFN-γ-regulated gene expression via mechanism(s) independent of STAT1 phosphorylation.

Figure 5.7 Effect of BRL49653 and 15d-PGJ2 on the IFN-γ-induced phosphorylation of STAT1 on Ser727 and Tyr701

PMA-differentiated THP-1 macrophages were pre-treated for 2h with BRL49653 (A) and 15d-PGJ_2 (B) at three different concentrations as indicated, or DMSO (DM, equal volume as the highest concentration of the respective agonist) as a vehicle control. The cells were then cultured for 30min in the presence or the absence of IFN-γ. Cell protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with each type of agonist versus IFN-γ-treated, DMSO control sample (N/S, not significant).


(A)
BRL49653
- + + + + -
IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
pSTAT1-Tyr701
Relative Expression
N/S N/S N/S
DM 20 DM 5 10 20 (μM)
IFN-γ - - + + + +
(B)
15d-PGJ2
- + + + + -
IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
pSTAT1-Tyr701
Relative Expression
N/S N/S N/S
DM DM 1 5 10 10 (μM)
IFN-γ - + + + + -

5.3.3 Effects of PPAR-γ agonists on the IFN-γ-induced STAT1 phosphorylation in human primary monocyte-derived macrophages

To rule out the possibility that the absence of an effect of PPAR-γ agonists on the IFN-γ-mediated STAT1 phosphorylation is a peculiar property of the differentiated THP-1 cell line, the effect of these agonists on STAT1 phosphorylation in human primary monocyte-derived macrophages was investigated.

Primary monocyte-derived macrophages were prepared as described in Chapter 4 (section 4.3.2). These cells were pre-treated with BRL49653 (20μM) or 15d-PGJ2 (10μM) for 2h prior to further incubation for 30min in the presence or the absence of IFN-γ. Cellular protein was then subjected to western blot analysis. Densitometric and statistical analysis of the data was carried out as detailed before. Consistent with previous results in THP-1 macrophages (Figure 5.7), neither of these ligands was found to affect the IFN-γ-induced STAT1 phosphorylation in human primary monocyte-derived macrophages (Figure 5.8).

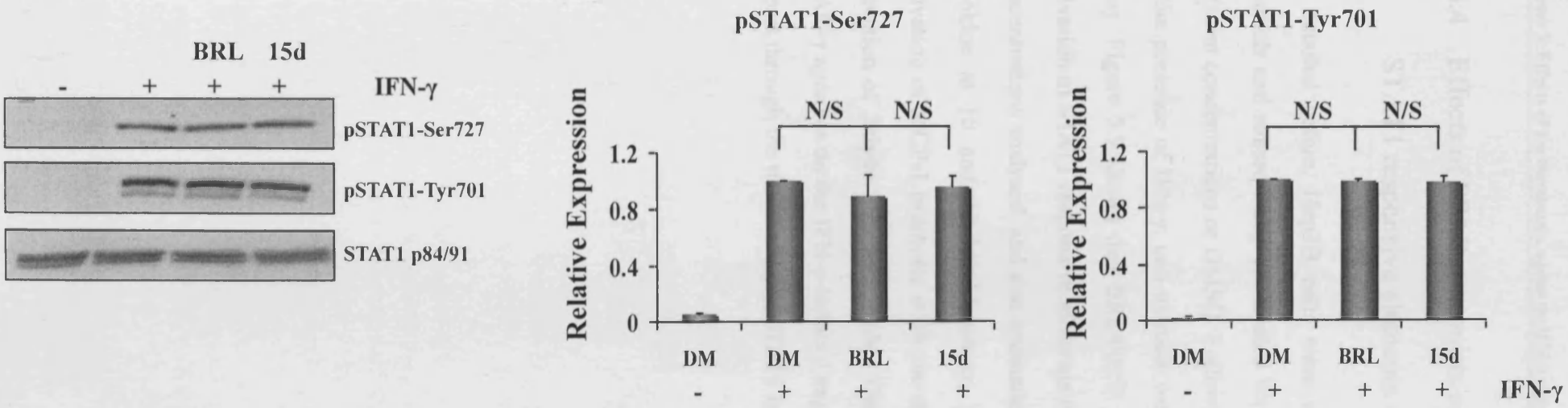


Figure 5.8 Effect of BRL49653 (BRL) and 15d-PGJ2 (15d) on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701 in human monocyte-derived macrophages

Human monocyte-derived macrophages were pre-treated for 2h with BRL49653 (20μM) or 15d-PGJ_2 (10μM) or DMSO (DM) as a vehicle control. The cells were then incubated for 30min in the presence or the absence of IFN-γ. Cell protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91 (A). The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with each type of agonist versus IFN-γ-treated, DMSO control sample (N/S, not significant).

5.3.4 Effects of PPAR-γ agonists on the IFN-γ-induced activation of STAT1 responsive elements and MCP-1 gene promoter

As detailed before, Hep3B cells were transfected with 3xly6e or MCP[213]Luc plasmids and subsequently pre-treated for 2h with BRL49653 or 15d-PGJ2 at three different concentrations or DMSO. Following further incubation for 6h in the absence or the presence of IFN-γ, cell extracts were prepared for luciferase reporter activity assay. Figure 5.9 shows that BRL49653 significantly inhibited the IFN-γ-mediated activation of STAT1 responsive elements (GAS) in the 3xly6e plasmid at all the three concentrations analysed, and also attenuated the activation of MCP-1 promoter by this cytokine at 10 and 20μM. Moreover, 15d-PGJ2 was also found to inhibit the activation of MCP-1 promoter at all the three concentrations used and to repress the activation of 3xly6e at 5 and 10μM. These results support the inhibitory effect of PPAR-γ agonists on the IFN-γ-induced expression of genes, including MCP-1, at least in part through the modulation of STAT1 transcriptional activity.

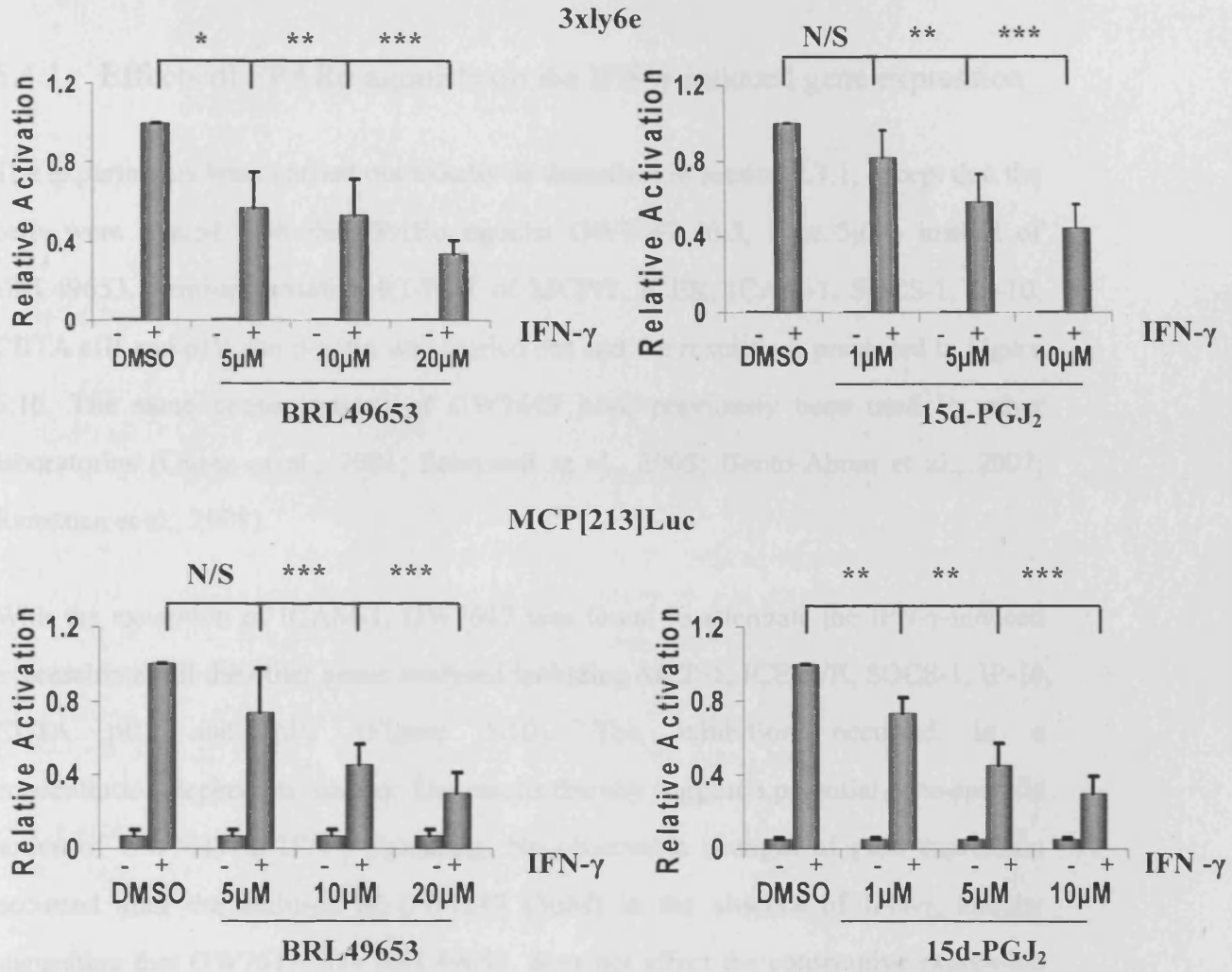


Figure 5.9 Effect of BRL49653 and 15d-PGJ2 on the activation of STAT1 responsive elements and MCP-1 promoter activity by IFN-γ

Hep3B cells were transfected with plasmids 3xly6e or MCP[213]Luc. Following 2h incubation with the respective agonist at three different concentrations or DMSO, transfected cells were treated with IFN-γ or left untreated for 6h after which cell extracts were prepared for luciferase reporter activity assay. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated controls, from three independent experiments. The data was further normalised to the relative activation of IFN-γ-treated DMSO control sample (arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with PPAR-γ agonist versus IFN-γ-stimulated DMSO control sample (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; N/S, not significant)

5.4 Effects of PPARα agonists on IFN-γ signalling

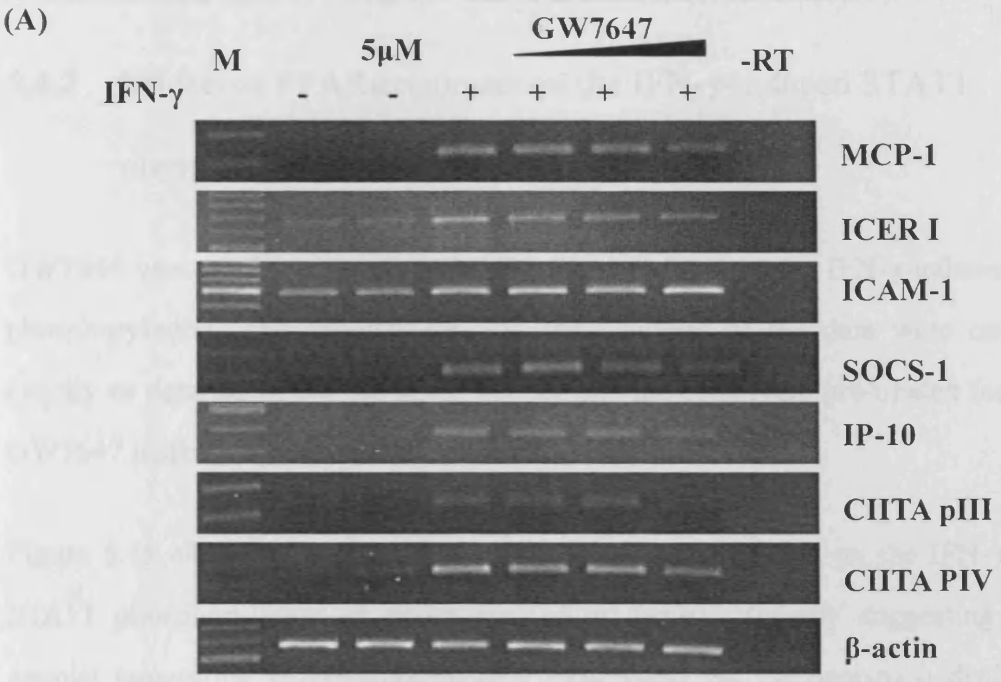
5.4.1 Effects of PPARα agonists on the IFN-γ-induced gene expression

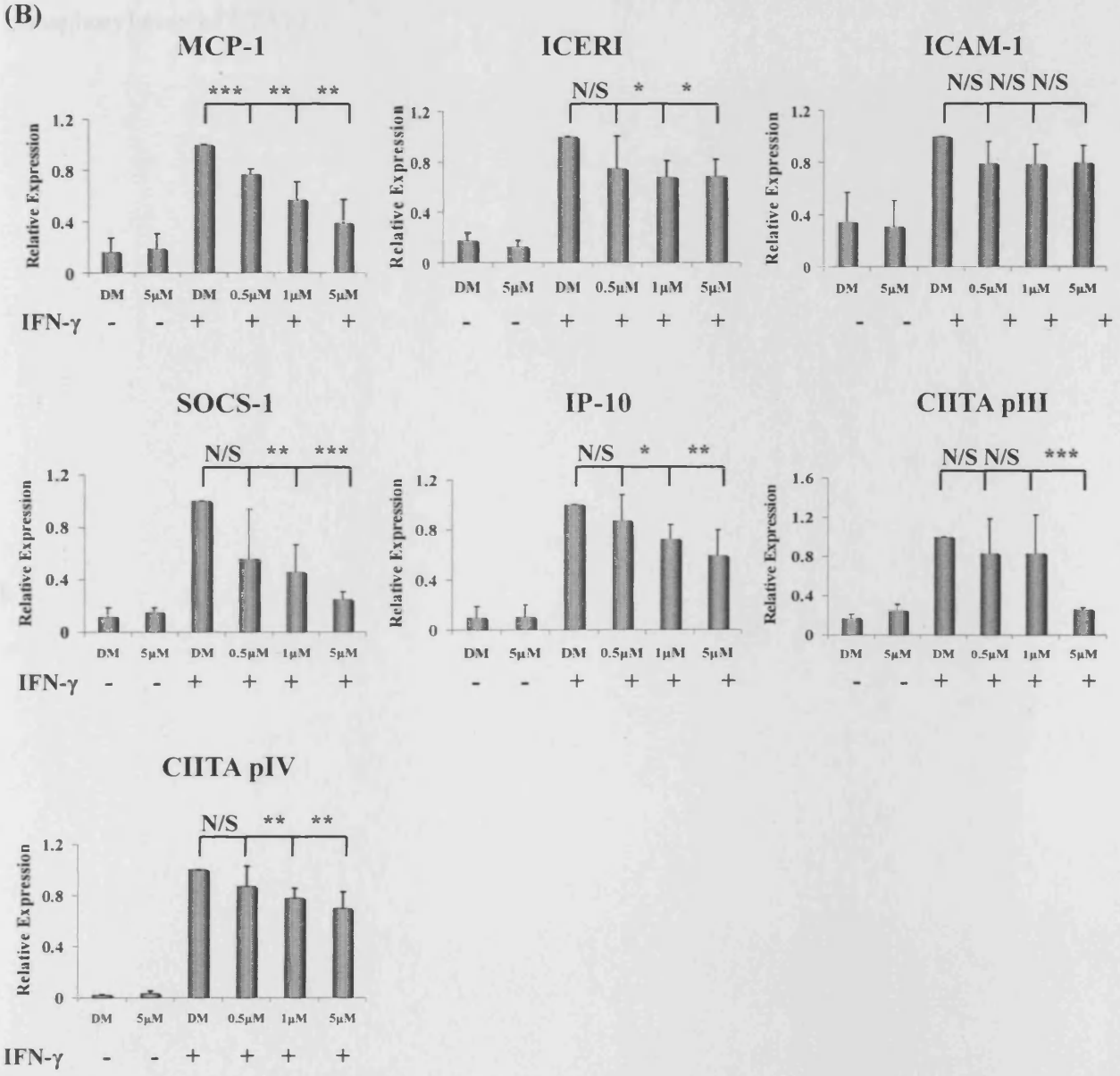
The experiments were carried out exactly as described in section 5.3.1, except that the cells were treated with the PPARα agonist GW7647 (0.5, 1 or 5μM) instead of BRL49653. Semi-quantitative RT-PCR of MCP-1, ICER, ICAM-1, SOCS-1, IP-10, CIITA pIII and pIV and β-actin was carried out and the results are presented in Figure 5.10. The same concentrations of GW7647 have previously been used by other laboratories (Gupta et al., 2001; Seimandi et al., 2005; Bento-Abreu et al., 2007; Ramanan et al., 2008).

With the exception of ICAM-1, GW7647 was found to attenuate the IFN-γ-induced expression of all the other genes analysed including MCP-1, ICERI/II, SOCS-1, IP-10, CIITA pIII and pIV (Figure 5.10). The inhibition occurred in a concentration-dependent manner. The results thereby suggest a potential gene-specific action of GW7647 in IFN-γ signalling. No observable changes of gene expression occurred after the addition of GW7647 (5μM) in the absence of IFN-γ, thereby suggesting that GW7647, like BRL49653, does not affect the constitutive expression of genes analysed in THP-1 macrophages.

Figure 5.10 Effect of GW7647 on the IFN-γ-induced mRNA expression of genes

PMA-differentiated THP-1 macrophages were pre-treated for 2h with GW7647 (0.5, 1 or 5μM) or DMSO (DM) as a vehicle control. The cells were then cultured for 3h in the presence or the absence of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1, ICER, ICAM-1, SOCS-1, IP-10, CIITA pIII, CIITA pIV and β-actin. The amplification products were analysed by agarose gel electrophoresis and the images shown are representative of three independent experiments (A). M denotes the 100bp DNA ladder (Appendix I); –RT indicates a reaction in which no reverse transcriptase was used during the cDNA synthesis step (RNA was from cells incubated with DMSO and IFN-γ). Panel B shows the relative expression (mean ±SD) of the indicated gene normalised to the expression of β-actin as determined by densitometric analysis from three independent experimental series (IFN-γ-treated, DMSO control assigned as 1). Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with GW7647 and IFN-γ (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; N/S, not significant).


(A)
M
5μM
GW7647
-RT
IFN-γ - - + + + +
MCP-1
ICER I
ICAM-1
SOCS-1
IP-10
CIITA pIII
CIITA PIV
β-actin


(B)
MCP-1
ICERI
ICAM-1
SOCS-1
IP-10
CIITA pIII
CIITA pIV
Relative Expression
DM 5μM DM 0.5μM 1μM 5μM
IFN-γ - - + + + +
*** ** **
N/S * *
N/S N/S N/S
N/S ** ***
N/S * **
N/S N/S ***
N/S ** **

5.4.2 Effects of PPARα agonists on the IFN-γ-induced STAT1 phosphorylation

GW7646 was employed again to investigate its effect on the IFN-γ-induced STAT1 phosphorylation. The experiments and the analyses of the data were carried out exactly as detailed in section 5.3.2, except that the cells were pre-treated for 2h with GW7647 instead of BRL49653.

Figure 5.11 clearly demonstrates that GW7647 had no effect on the IFN-γ-induced STAT1 phosphorylation at either Ser727 or Tyr701, thereby suggesting that the agonist targets the IFN-γ-induced gene expression via mechanism(s) distinct from phosphorylation of STAT1.

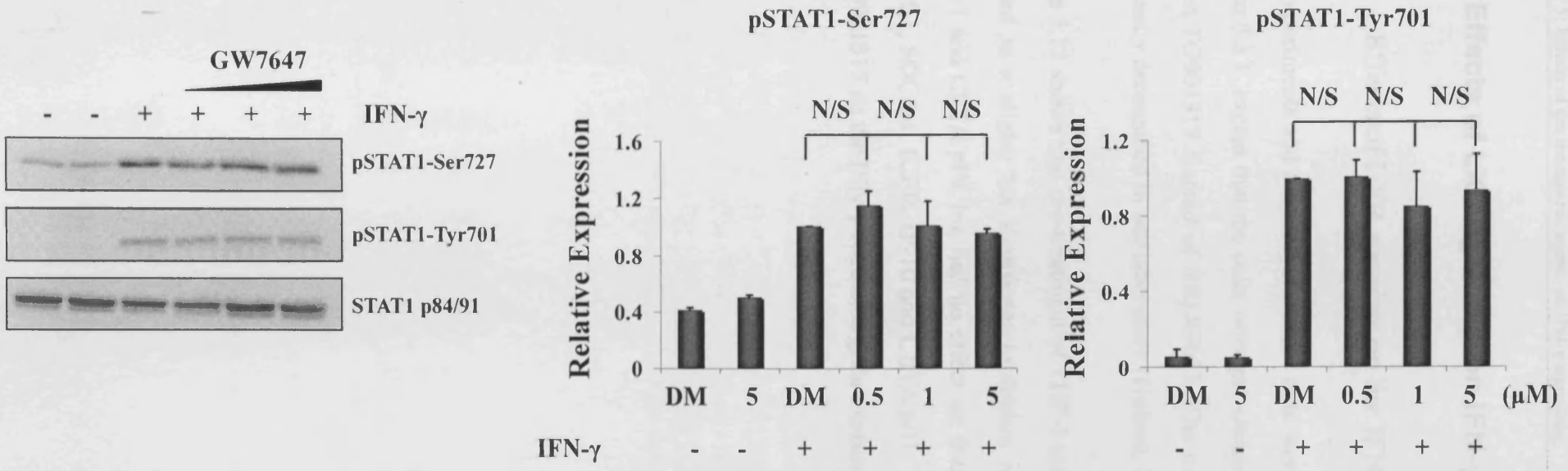


Figure 5.11 Effect of GW7647 on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 cells were pre-treated for 2h with GW7647 at three different concentrations as indicated or DMSO (equal volume as GW7647 at 5μM) as a vehicle control. The cells were then cultured for 30min in the presence or the absence of IFN-γ. Cell protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from the IFN-γ-treated, DMSO control sample was arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with GW7647 versus IFN-γ-treated, DMSO control sample (N/S, not significant).

5.5 Effects of LXR agonists on IFN-γ signalling

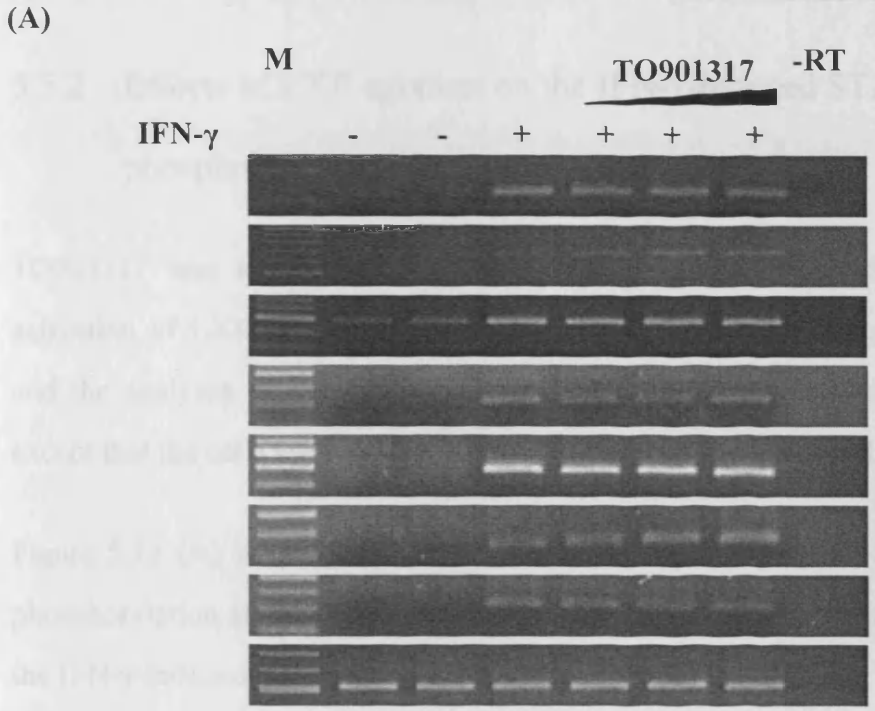
5.5.1 Effects of LXR agonists on the IFN-γ-induced gene expression

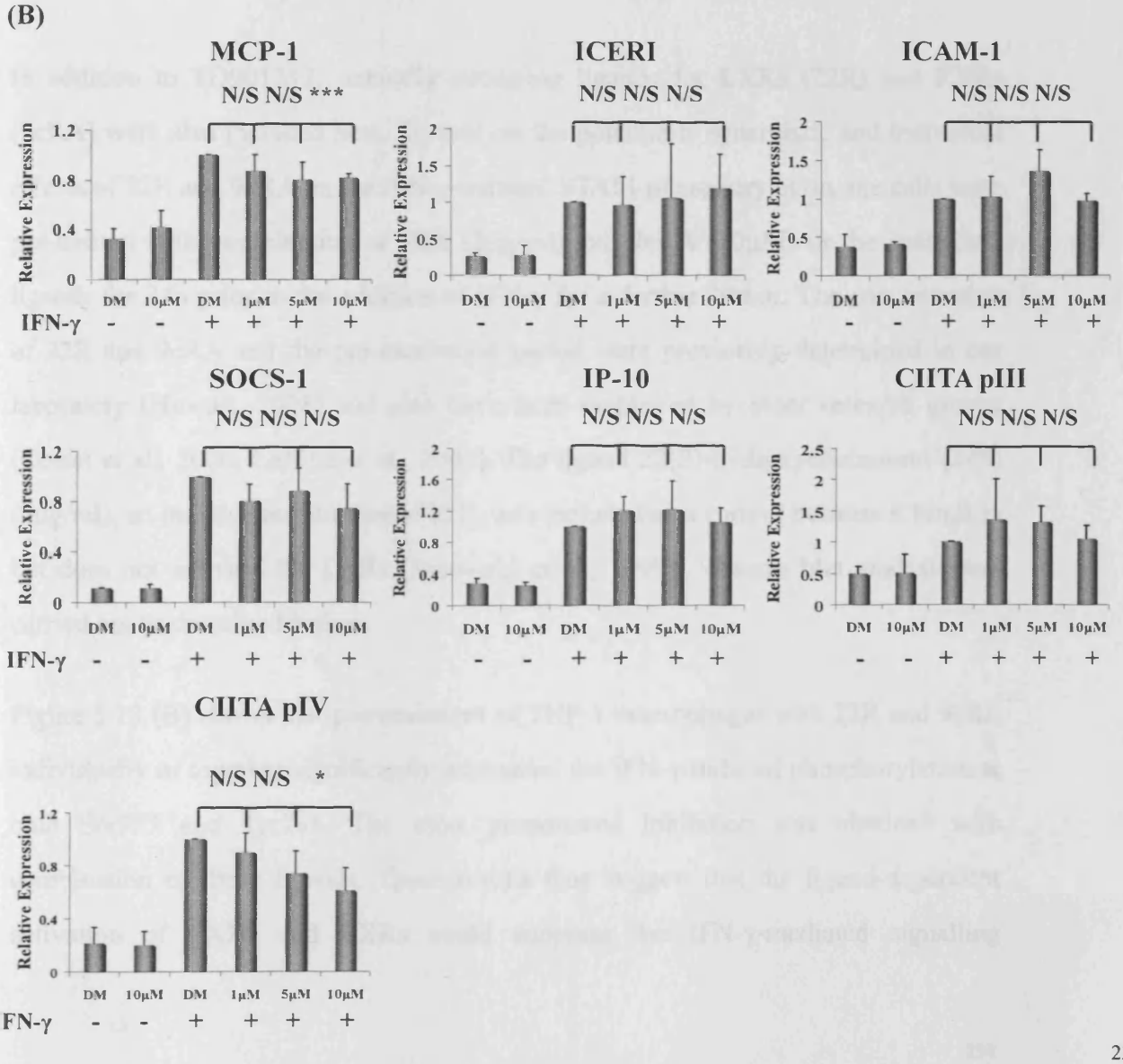
The experiments and the analyses of the data were carried out exactly as described in section 5.3.1, except that the cells were pre-treated for 2h with the non-steroidal LXR agonist TO901317 instead of BRL49653. The concentration of TO901317 has been previously determined in our laboratory (Huwait, 2008).

Figure 5.12 shows that pre-treatment of THP-1 macrophages with TO901317 at 10μM resulted in a slight, but significant inhibition of the IFN-γ-induced expression of MCP-1 and CIITA pIV, but had no effect on that of other genes analysed, including ICAM-1, SOCS-1, ICER, IP-10 and CIITA pIII. The results suggest a selective effect of TO901317 on the IFN-γ-regulated gene expression.

Figure 5.12 Effect of TO901317 on the IFN-γ-induced mRNA expression of genes

PMA-differentiated THP-1 macrophages were pre-treated for 2h with TO901317 (1, 5 or 10μM) or DMSO as a vehicle control. The cells were then cultured for 3h in the presence or the absence of IFN-γ. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1, ICER, ICAM-1, SOCS-1, IP-10, CIITA pIII, CIITA pIV and 28S rRNA. The amplification products were analysed by agarose gel electrophoresis and the images shown are representative of three independent experiments (A). M denotes the 100bp DNA ladder (Appendix I); –RT denotes a reaction in which no reverse transcriptase was present during the cDNA synthesis step (RNA was from cells incubated with DMSO and IFN-γ). Panel B shows the relative expression (mean ±SD) of the indicated gene normalised to the expression of β-actin as determined by densitometric analysis from three independent experimental series (IFN-γ-treated, DMSO control assigned as 1). Statistical comparisons were carried out using the Student's *t* test between the DMSO and IFN-γ treated control and samples under treatment with TO901317 and IFN-γ (* $P<0.05$; *** $P<0.001$; N/S, not significant).


(A)
M
TO901317
-RT
IFN-γ - - + + + +
MCP-1
ICER I
ICAM-1
SOCS-1
IP-10
CIITA pIII
CIITA pIV
28S rRNA


(B)
MCP-1
N/S N/S ***
ICERI
N/S N/S N/S
ICAM-1
N/S N/S N/S
SOCS-1
N/S N/S N/S
IP-10
N/S N/S N/S
CIITA pIII
N/S N/S N/S
CIITA pIV
N/S N/S *
Relative Expression
DM 10μM DM 1μM 5μM 10μM
IFN-γ - - + + + +

5.5.2 Effects of LXR agonists on the IFN-γ-induced STAT1 phosphorylation

TO901317 was again employed here to analyse the effect of ligand-dependent activation of LXRs on the IFN-γ-induced STAT1 phosphorylation. The experiment and the analyses of the data were carried out exactly as detailed in section 5.3.2, except that the cells were pre-treated for 2h with TO901317 instead of BRL49653.

Figure 5.13 (A) shows that TO901317 had no effect on the IFN-γ-induced STAT1 phosphorylation at either Ser727 or Tyr701, thereby suggesting that the agonist targets the IFN-γ-induced gene expression via mechanism(s) distinct from phosphorylation of STAT1 at Ser727 and Tyr701.

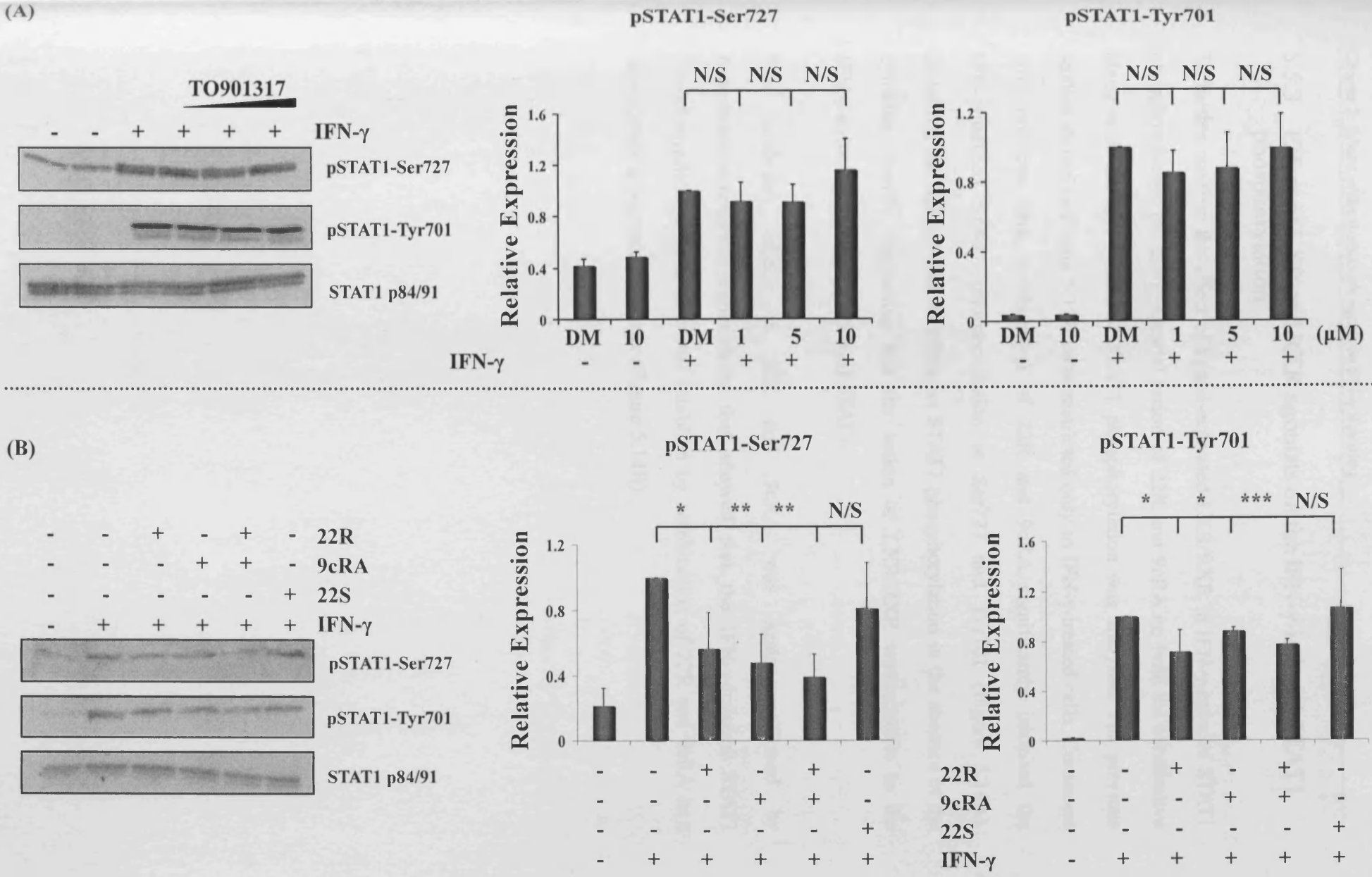
In addition to TO901317, naturally occurring ligands for LXRs (22R) and RXRs (9cRA) were also included here. To analyse the potentially synergistic and individual effects of 22R and 9cRA on the IFN-γ-induced STAT1 phosphorylation, the cells were pre-treated with combination of 22R (2μg/ml) and 9cRA (10μM) or the individual ligands for 24h prior to the addition of IFN-γ for a further 30min. The concentration of 22R and 9cRA and the pre-incubation period were previously determined in our laboratory (Huwait, 2008) and also have been employed by other research groups (Costet et al., 2000; Laffitte et al., 2001). The ligand 22(S)-hydroxycholesterol (22S) (2μg/ml), an inactive enantiomer of 22R, was included as a control because it binds to but does not activate the LXRs (Janowski et al., 1999). Western blot analysis was carried out as described before.

Figure 5.13 (B) shows that pre-treatment of THP-1 macrophages with 22R and 9cRA individually or together significantly attenuated the IFN-γ-induced phosphorylation at both Ser727 and Tyr701. The most pronounced inhibition was obtained with combination of these ligands. These results thus suggest that the ligand-dependent activation of LXRs and RXRs could suppress the IFN-γ-mediated signalling

individually or in combination. Pre-treatment of 22S showed no effect on the IFN-γ-induced phosphorylation, thereby suggesting that the observed effect by 22R was specific to LXR activation.

Figure 5.13 Effect of TO901317, 22R, 9cRA and 22R+9cRA on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 cells were pre-treated for 2h with TO901317 (1, 5 or 10μM), 24h with 22R (2μg/ml), 9cRA (10μM), 22R+9cRA, or DMSO (DM, equal volume as highest concentration of respective agent) as a vehicle control. 22S (2μg/ml) was included as a negative control for 22R. The cells were then cultured for 30min in the presence or the absence of IFN-γ. Cell protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with each type of ligand versus IFN-γ-treated, DMSO control sample (**P*<0.05; ***P*<0.01; ****P*<0.001; N/S, not significant).


(A)
TO901317
- - + + + + IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
pSTAT1-Tyr701
N/S N/S N/S
Relative Expression
1.6
1.2
0.8
0.4
0
DM 10 DM 1 5 10
IFN-γ - - + + + +
N/S N/S N/S
Relative Expression
1.2
0.8
0.4
0
DM 10 DM 1 5 10 (μM)
- - + + + +
(B)
- - + - + - 22R
- - - + + - 9cRA
- - - - - + 22S
- + + + + + IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
* ** ** N/S
Relative Expression
1.2
0.8
0.4
0
- - + - + -
- - - + + -
- - - - - +
- + + + + +
pSTAT1-Tyr701
* * *** N/S
Relative Expression
1.6
1.2
0.8
0.4
0
22R - - + - + -
9cRA - - - + + -
22S - - - - - +
IFN-γ - + + + + +

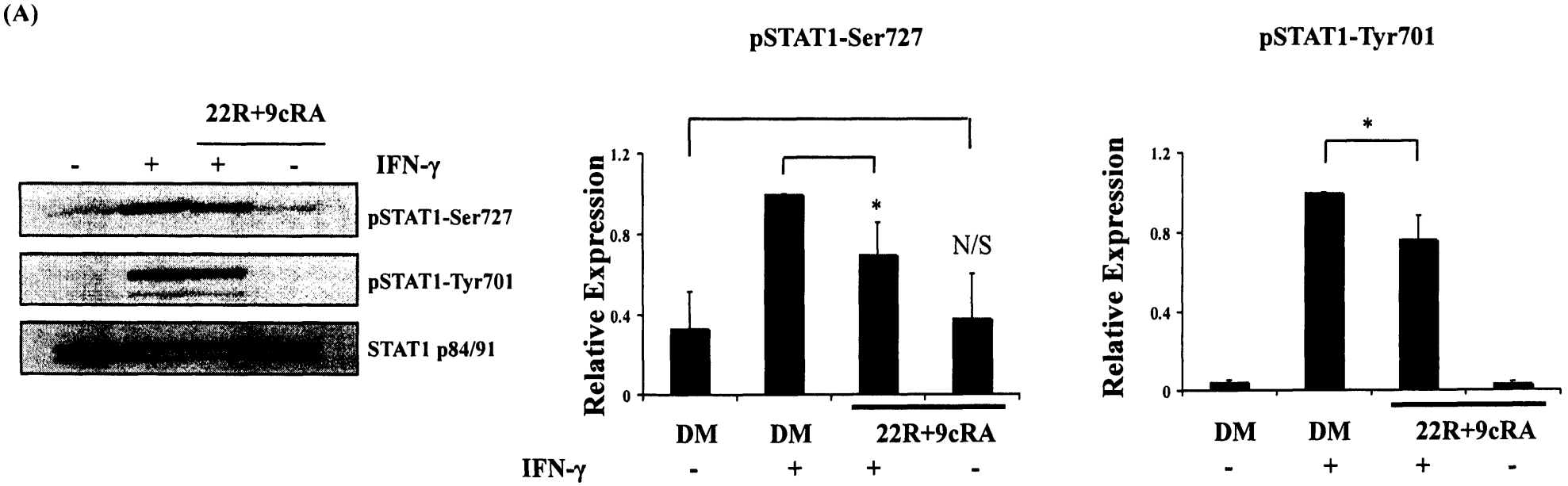
5.5.3 Effect of LXR and RXR agonists on the IFN-γ-induced STAT1 phosphorylation

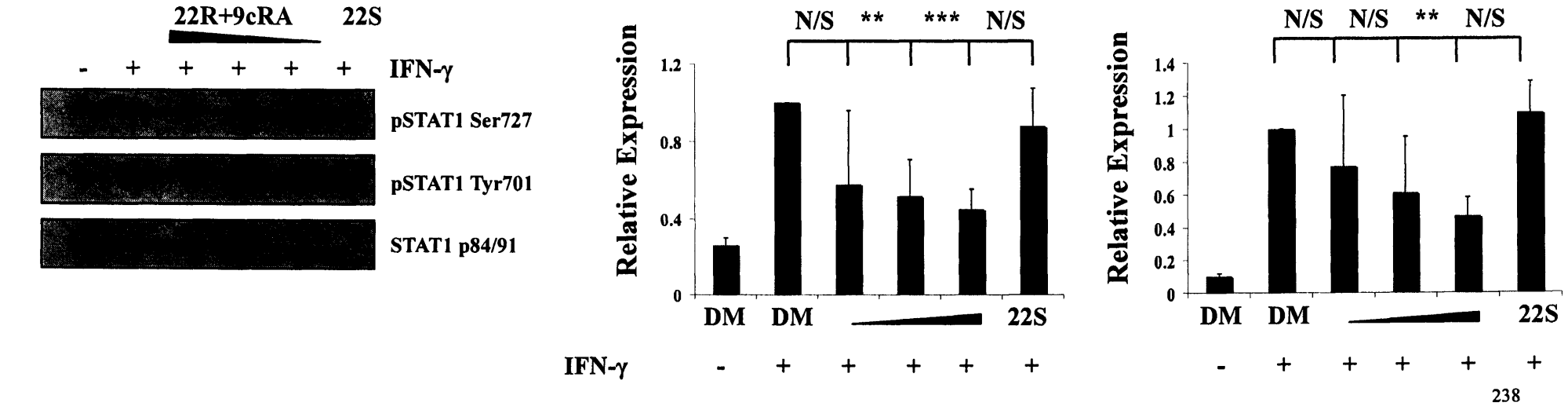
To further confirm the effect of ligand-activated LXR/RXR in IFN-γ-induced STAT1 phosphorylation, the combinatorial action of 22R and 9cRA on both the constitutive along with the IFN-γ-induced STAT1 phosphorylation was analysed. The previous studies shown in Figure 5.13B were restricted only to IFN-γ-treated cells. Consistent with previous data, combination of 22R and 9cRA significantly inhibited the IFN-γ-induced STAT1 phosphorylation at Ser727 and Tyr701 (Figure 5.14A). However, the ligands had no effect on STAT1 phosphorylation in the absence of the cytokine, thereby suggesting that the action of LXR/RXR was specific to the IFN-γ-inducible phosphorylation of STAT1.

The inhibitory effect of 22R and 9cRA was again confirmed by concentration-response experiments that showed that the IFN-γ-induced STAT1 phosphoryaltion at both sites was inhibited by combination of 22R and 9cRA in a concentration-dependent manner (Figure 5.14B).

Figure 5.14 Effect of 22R and 9cRA on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701

PMA-differentiated THP-1 cells were pre-treated for 24h with 22R (2μg/ml) and 9cRA (10μM) (A), or 22R (1, 2 or 5μg/ml) and 9cRA (10μM) (B) or DMSO (equal volume as the highest concentration of the respective agents) as a vehicle control. 22S (2μg/ml) was included as a negative control for 22R. The cells were then cultured for 30min in the presence or the absence of IFN-γ. Cell protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with the agonists versus IFN-γ-treated, DMSO control sample (**P*<0.05; ***P*<0.01; ****P*<0.001; N/S, not significant).


(A)
22R+9cRA
- + + - IFN-γ
pSTAT1-Ser727
pSTAT1-Tyr701
STAT1 p84/91
pSTAT1-Ser727
Relative Expression
1.2
0.8
0.4
0
*
N/S
DM DM 22R+9cRA
IFN-γ - + + -
pSTAT1-Tyr701
Relative Expression
1.2
0.8
0.4
0
*
DM DM 22R+9cRA
- + + -


(B)
22R+9cRA 22S
- + + + + + IFN-γ
pSTAT1 Ser727
pSTAT1 Tyr701
STAT1 p84/91
pSTAT1-Ser727
Relative Expression
1.2
0.8
0.4
0
N/S ** *** N/S
DM DM 22S
IFN-γ - + + + + +
pSTAT1-Tyr701
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N/S N/S ** N/S
DM DM 22S
- + + + + +

5.5.4 Effects of LXR and RXR agonists on the IFN-γ-induced STAT1 phosphorylation in human primary monocyte-derived macrophages

We next decided to confirm the action of LXR/RXR on the IFN-γ-induced STAT1 phosphorylation in human primary monocyte-derived macrophages to rule out the possibility that the effect of 22R and 9cRA observed previously (Figures 5.13 and 5.14) was a peculiar property of the differentiated THP-1 cell line. The human primary macrophages were prepared and the experiments were carried out as described in section 5.3.3 except that the cells were pre-treated for 24h with combination of 22R (2μg/ml) and 9cRA (10μM) instead of the PPARγ agonists for 2h. Statistical analysis of the data was performed as detailed before.

Figure 5.15 shows that pre-treatment of the cells with combination of 22R and 9cRA significantly attenuated the IFN-γ-induced STAT1 phosphorylation in human monocyte-derived macrophages, thereby suggesting that the inhibitory effect of 22R with 9cRA on IFN-γ signalling was not specific to the THP-1 cell line but probably a general property to human macrophages.

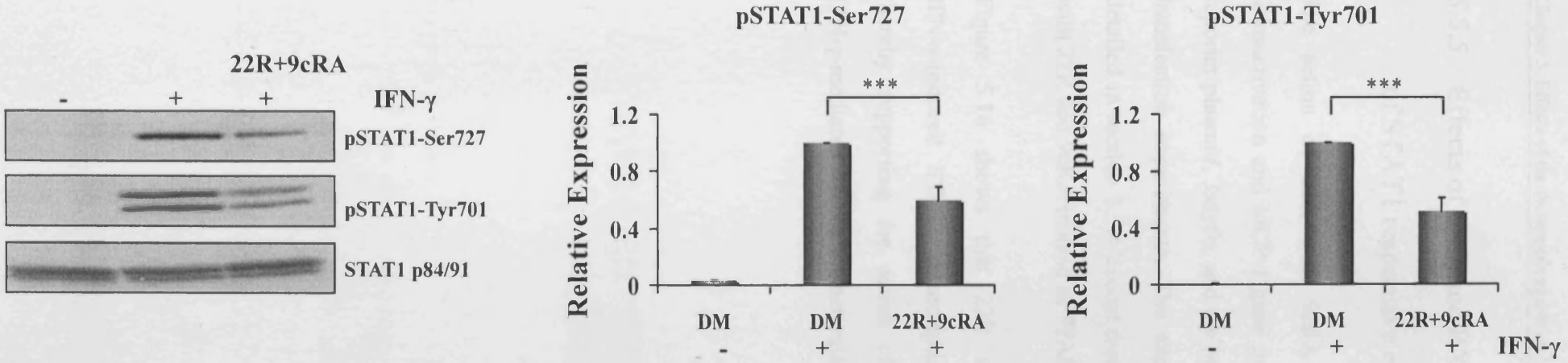


Figure 5.15 Effect of 22R with 9cRA on the IFN-γ-induced phosphorylation of STAT1 at Ser727 and Tyr701 in human monocyte-derived macrophages

Human monocyte-derived macrophages were pre-treated for 24h with 22R (2μg/ml) with 9cRA (10μM) or DMSO as a vehicle control. The cells were then cultured for 30min in the presence or the absence of IFN-γ. Cell protein extracts were prepared using the Laemmli sample buffer and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, pSTAT1 Ser727 and STAT1 p84/91. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at each of these sites normalised to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experimental series (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with ligands versus IFN-γ-treated, DMSO control sample (***$P<0.001$).

5.5.5 Effects of LXR and RXR agonists on the IFN-γ-induced activation of STAT1 responsive elements and MCP-1 gene promoter

The action of 22R with 9cRA on the IFN-γ-mediated activation of STAT1 transactivation and MCP-1 gene promoter was also confirmed using GAS-regulated reporter plasmid, 3xly6e, and the MCP-1 promoter reporter plasmid MCP[213]Luc in transfection experiments. The experiments and data analysis were performed as detailed in section 5.3.4 except that the transfected Hep3B cells were treated for 24h with 22R and 9cRA instead of PPARγ agonists for 2h.

Figure 5.16 shows that 22R and 9cRA together significantly suppress the IFN-γ-induced STAT1 transcriptional activity and MCP-1 promoter activation, thereby supporting the action of ligand-dependent activation of LXR/RXR on IFN-γ-mediated STAT1 tranactivation and MCP-1 transcription.

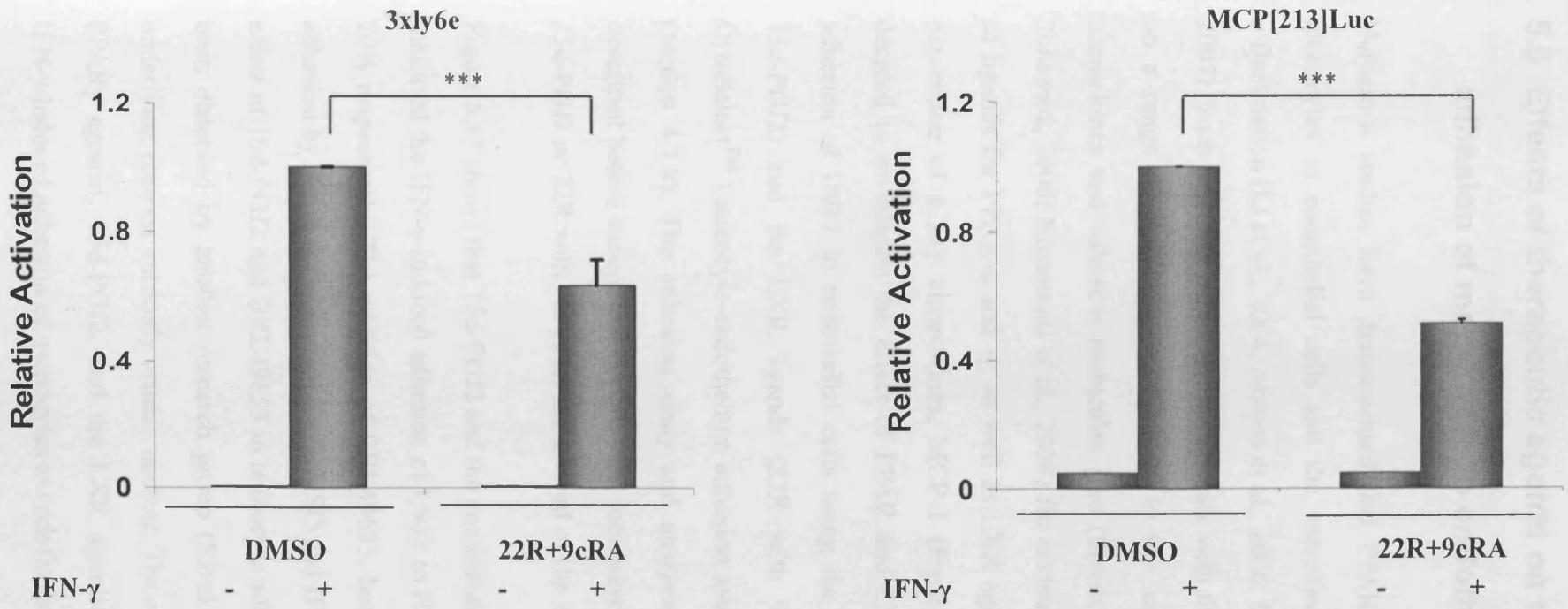


Figure 5.16 Effect of 22R+9cRA on the IFN-γ-stimulated activation of STAT1 responsive elements and MCP-1 gene promoter

Hep3B cells were transfected with plasmids 3xly6e or MCP[213]Luc. Following 24h incubation with combination of 22R (2μg/ml) and 9cRA (10μM) or DMSO, transfected cells were treated with IFN-γ or left untreated for 6h after which cell extracts were prepared for luciferase reporter activity assay. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated samples, from three independent experiments. The data was further normalised to the relative activation of IFN-γ-treated DMSO control sample (arbitrarily assigned as 1). Student's *t* test was carried out on samples treated with agonists versus IFN-γ-stimulated DMSO control (***$P<0.001$).

5.6 Effects of therapeutic agents on the IFN-γ-induced adhesion of monocytes to endothelial cells

Numerous studies have demonstrated that PPAR ligands inhibit the adhesion of leukocytes to endothelial cells and the recruitment of leukocytes to the site of inflammation (Li et al., 2004; Ahmed et al., 2006; Lee et al., 2007a; Straus and Glass, 2007). Such an effect is thought to correlate with the transrepression action of PPARs on a range of molecules contributing to the adhesion and recruitment, such as chemokines and adhesion molecules [see (Straus and Glass, 2007; Bensinger and Tontonoz, 2008; Rigamonti et al., 2008) for reviews]. Our results show that addition of ligands for PPAR-α and -γ, as well as LXR agonists suppress the IFN-γ-induced expression of a key chemokines, MCP-1 (Figures 5.6, 5.10 and 5.12). We thus decided to investigate the effect of PPAR and LXR ligands on the IFN-γ-induced adhesion of U937 to endothelial cells using the PPARγ agonists (BRL49653 and 15d-PGJ2) and the LXR ligands (22R with 9cRA). We again employed the CytoSelect™ Leukocyte-endothelium adhesion assay system as detailed in Chapter 4 (section 4.3.9). The adhesion assay and analysis of the data was carried out as described before except that the HUVEC monolayer was pre-treated with BRL49653, 15d-PGJ2 or 22R with 9cRA for 2h, instead of the inhibitors for 1h.

Figure 5.17 shows that 15d-PGJ2 and the combination of 22R with 9cRA significantly inhibited the IFN-γ-induced adhesion of U937 to HUVEC by approximately 50% and 20% respectively. The addition of BRL49653, however, resulted in an induction of adhesion by about 60% compared to DMSO and IFN-γ-treated control. Such opposite effect of 15d-PGJ2 and BRL49653 in leukocyte adhesion to endothelial cells has also been observed by another research group (Rival et al., 2002), although the exact underlying reasons currently remain unclear. The results in isolation suggest that the PPARγ agonist, 15d-PGJ2, and the LXR agonists, 22R and 9cRA, inhibited the IFN-γ-induced adhesion of monocytes to endothelial cells.

It is noteworthy that the cellular viability assays (ViaLight and MTT assays) carried out on HUVECs (presented as black and blue lines in Figure 5.17) showed that incubation with the agonists and the cytokine for 32h (treatment with agonists for 2h plus with IFN-γ for another 30h) resulted in a low HUVEC viability. The data suggests that the low HUVEC viability could also contribute to the decreased adhesion of U937 to HUVEC caused by the agonists 15d-PGJ2 along with 22R and 9cRA. However, although the addition of BRL49653 seemed to result in as much HUVEC cell death as the other agonists, the adhesion of U937 to HUVEC was induced. It is therefore difficult to come to any firm conclusions on whether the reduced adhesion by the agonists could be attributed to their actions on the IFN-γ-mediated cellular inflammatory response, or simply be due to reduced HUVEC viability.

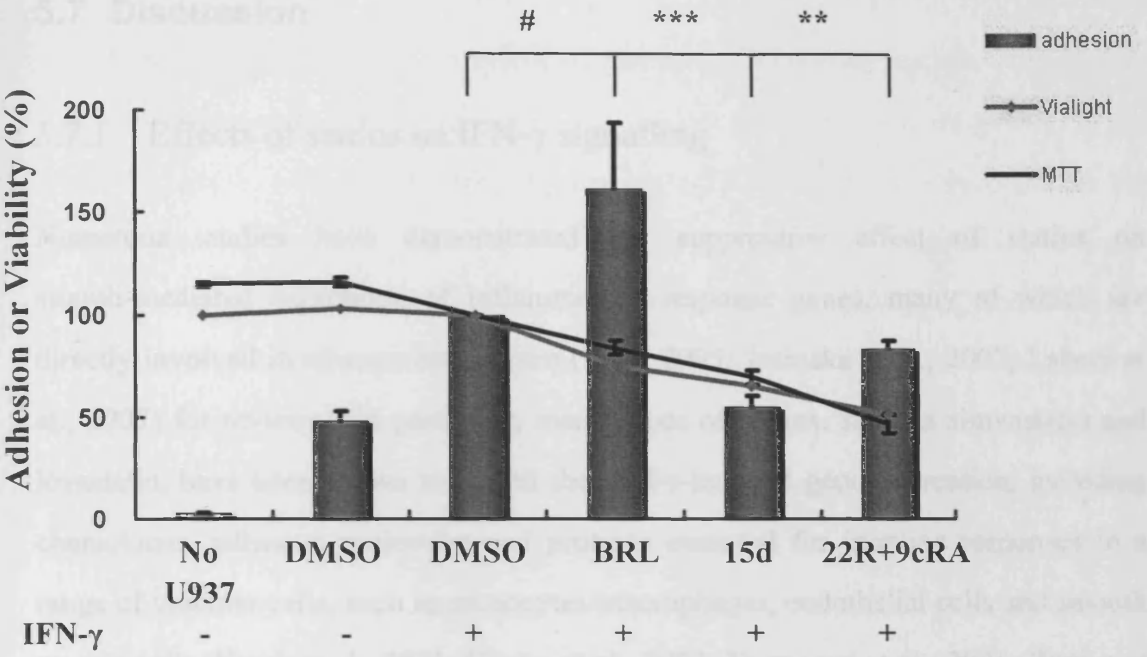


Figure 5.17 The effects of therapeutic drugs on the IFN-γ-induced adhesion between U937 and HUVEC and analysis of viability of HUVEC under variety of treatments

Monolayers of HUVEC were pre-treated with BRL49653 (20μM), 15d-PGJ2 (10 μM) or 22R (2μg/ml) with 9cRA (10μM) for 2h and further stimulated with IFN-γ (1000U/ml) for 30h. LeukoTracker™-labelled U937 cells were added and allowed to attach to the HUVEC monolayer for 1h. Adherent cells were lysed and quantified by fluorescence measurement at 485nm/520nm. HUVEC monolayers without treatment, or addition of U937 cells (No U937) were used as negative controls. Statistical analysis was performed from three independent experiments. Blue bars show the percentage of adhesion (mean±SD) under variety of treatments relative to DMSO (DM) plus IFN-γ-treated control (assigned as 100%). Student's *t* test was carried out on ligands treated samples versus IFN-γ treated DMSO control sample (significance of induction: #, $P<0.05$; significance of inhibition: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; N/S, not significant). Green and black lines in the figure show the viability of HUVEC under various treatments using ViaLight assay and MTT assays respectively. The value from HUVEC with DMSO and IFN-γ treated sample was assigned as 100% viable. Results of MTT assay (mean±SD) are representative of three independent experiments. Luminescence reading of ViaLight assay was obtained from single experiment.

5.7 Discussion

5.7.1 Effects of statins on IFN-γ signalling

Numerous studies have demonstrated the suppressive effect of statins on stimuli-mediated expression of inflammatory response genes, many of which are directly involved in atherosclerosis [see (Liao, 2005; Jasinska et al., 2007; Lahera et al., 2007) for reviews]. In particular, many types of statins, such as simvastatin and lovastatin, have been shown to inhibit the IFN-γ-induced gene expression, including chemokines, adhesion molecules and proteins essential for immune responses in a range of vascular cells, such as monocytes/macrophages, endothelial cells and smooth muscle cells (Kwak et al., 2001; Chung et al., 2002; Townsend et al., 2004; Buttice et al., 2006; Montecucco et al., 2009) (see Table 1.9 for a list of studies). Consistent with these previous findings, work presented in this chapter clearly shows that pre-treatment of THP-1 macrophages with simvasatin inhibits the IFN-γ-induced expression of MCP-1, ICAM-1, CIITA pIV and SOCS-1 in a concentration-dependent manner, thereby suggesting an anti-inflammatory and immunomodulatory effect of simvastatin in human macrophages (Figure 5.3). In contrast to CIITA pIV, the expression of CIITA pIII was not affected by simvastatin, thereby suggesting a promoter-specific action of simvastatin on the IFN-γ-induced CIITA expression. Several studies have demonstrated that simvastatin suppresses the IFN-γ-induced CIITA pIV expression in macrophages and endothelial cells (Kwak et al., 2001; Sadeghi et al., 2001; Mach, 2002a; Lee et al., 2008), while Buttice et al. (2006) showed that simvastatin did not affect either CIITA pIII or pIV in response to IFN-γ in human aortic smooth muscle cells. These results together suggest that simvastatin may repress the IFN-γ-stimulated expression of CIITA in a promoter- and cell-type-specific manner.

Figure 5.4B shows that simvastatin at concentrations that inhibited the IFN-γ-induced gene expression failed to attenuate STAT1 phosphorylation at either Ser727 or Tyr701,

thereby suggesting the involvement of a STAT1 phosphorylation-independent mechanism for the simvastatin-mediated repression. In addition, as shown in Figure 5.5, pre-treatment of the cells with simvastatin resulted in a decrease in the IFN-γ-induced luciferase expression regulated by STAT1 consensus sites (GAS) in the 3xly6e vector and the activation of MCP-1 gene promoter. As summarised in Table 5.2, simvastatin inhibits the IFN-γ-induced expression of MCP-1, ICAM-1, CIITA pIV and SOCS-1. As detailed before in sections 3.1.5 and 4.1.4, the expression of these genes has been shown to be mediated, at least in part, by a STAT1-dependent pathway in response to IFN-γ. These results therefore suggest that simvastatin probably inhibits the expression of these genes by attenuating the STAT1 transcriptional activity in response to IFN-γ, but without targeting STAT1 phosphorylation.

In contrast to simvastatin, lovastatin was found not to affect the IFN-γ-induced gene expression (Figure 5.2) or STAT1 phosphorylation (Figure 5.4A). Such differential effect of different types of statins has been previously documented. For example, Sugiyama et al. (2000) showed that simvastatin, but not pravastatin, induces the bone morphogenetic protein-2 expression in human osteosarcoma cells. Moreover, in contrast to our findings in THP-1 macrophages, Chung et al. (2002) and Townsend et al (2004) have shown that lovastatin suppresses the IFN-γ-induced expression of genes, such as ICAM-1, TNF-α and CD40, by attenuating STAT1 phosphorylation at Ser727 in endothelial, smooth muscle and microglial cells. These results together suggest that statins probably exert their anti-inflammatory effects in a cell type- and statin type-specific manner.

5.7.2 Effects of PPARγ agonists on IFN-γ signalling

As detailed in the General Introduction (section 1.8.1), inhibitory effects of PPAR agonists on the cytokine-stimulated inflammatory responses in vascular cells are well documented [see (Marx et al., 2004; Pascual and Glass, 2006; Ricote and Glass, 2007)

for reviews]. In particular, numerous studies have shown that the ligand-activated PPARγ represses the IFN-γ-induced expression of a large range of inflammatory response genes, such as chemokines (e.g. IP-10) and inflammatory mediator molecules (e.g. COX2) (see Table 1.7 for a list of IFN-γ-induced inflammatory response genes repressed by PPARs). Consistent with these findings, Figure 5.6 shows that pre-treatment of THP-1 macrophages with BRL49653 inhibited the IFN-γ-induced expression of MCP-1, IP-10, CIITA pIII and SOCS-1. It should be noted that both simvastatin and BRL49653 repressed the expression of MCP-1 and SOCS-1 in response to IFN-γ (Figure 5.2 and 5.6). Interestingly, the anti-inflammatory properties of statins have been previously shown to be mediated, at least in part, by activation of PPARγ, through a mechanism involving the generating of endogenous PPAR ligands (e.g. 15d-PGJ2) (Yano et al., 2007). It is thus tempting to speculate that the repressive effect of simvastatin on the IFN-γ-induced expression of MCP-1 and SOCS-1 is probably mediated by the action of PPARγ, although more detailed studies will be required to confirm this. Park et al. (2003) previously demonstrated that BRL49653 inhibited the IFN-γ-induced expression of MCP-1 and IP-10 by inducing the transcription of SOCS-1, and thus activating the SOCS-dependent negative regulation of IFN-γ signalling in mouse astrocytes. This is unlikely to be the case in THP-1 macrophages as the expression of SOCS-1 is inhibited by BRL49653, thereby suggesting an alternative mechanism underlying the repressive action of this agent.

A number of studies have previously shown that the PPARγ agonist, 15d-PGJ2, exerts its transrepression activity on the IFN-γ-mediated inflammatory gene expression by inhibiting STAT1 phosphorylation in mouse macrophage, mesangial cells and insulinoma cells (Chen et al., 2003; Weber et al., 2004; Panzer et al., 2008). We however found that in THP-1 and human primary monocyte-derived macrophages, both 15d-PGJ2 and BRL49653 have no effect on the IFN-γ-induced STAT1 phosphorylaiton at either Ser727 or Tyr701 (Figures 5.7 and 5.8). Therefore, the PPARγ agonits probably exert their anti-inflammatory effects through a cell type- and

potentially species-specific-mechanisms.

Table 5.2 Summary of the repressive effects of therapeutic agents on the IFN-γ-induced gene expression

	MCP-1	IP-10	MIP-1β	ICAM-1	CIITA pIII	CIITA pIV	SOCS-1	ICER
Lovastatin	→	→	/	→	/	/	→	→
Simvastatin	↓	/	→	↓	→	↓	↓	→
BRL49653	↓	↓	/	→	↓	→	↓	→
GW7647	↓	↓	/	→	↓	↓	↓	↓
TO901317	↓	→	/	→	→	↓	→	→

→, no effect; ↓, inhibition; /, not investigated

Abbreviations: MCP; monocyte chemoattractant protein; IP; interferon-gamma-induced protein; MIP; macrophage inflammatory protein; ICAM, intercellular adhesion molecule; CIITA; MHC class II activator; SOCS, suppressors of cytokine signaling; ICER; inducible cAMP early repressor

Although PPARγ agonists seem not to target STAT1 phosphorylation, both BRL49653 and 15d-PGJ2 attenuated the IFN-γ-induced activation of STAT1 response elements (3xly6e construct) and MCP-1 gene promoter (MCP[213]Luc) (Figure 5.9). As summarised in Table 5.2, the IFN-γ-inducible expression of MCP-1, IP-10 and SOCS-1 was also inhibited by addition of BRL49653. In addition, the expression of all these genes in response to IFN-γ has been previously shown to be mediated, at least in part, through a STAT1-dependent pathway. Taken together, these results suggest that BRL49653 attenuates the IFN-γ-induced expression of MCP-1, IP-10 and SOCS-1 by targeting STAT1 transcriptional activity without affecting STAT1 phosphorylation.

5.7.3 Effects of PPARα agonists on IFN-γ signalling

Figure 5.10 shows that the PPARα agonist GW7649 inhibited the IFN-γ-induced

expression of all the genes analysed except ICAM-1 (Table 5.2). Such a repressive activity of PPARα agonist on pro-inflammatory cytokine-induced gene expression in macrophages is consistent with some previous studies (Shu et al., 2000; Souissi et al., 2008). In addition, statins have been suggested to exert their pleiotropic effects, at least in part, through a PPARα-dependent pathway (Paumelle et al., 2006). It is worth noting that both simvastatin and GW7647 attenuated the IFN-γ-induced expression of MCP-1, IP-10, SOCS-1 and CIITA pIV, thereby suggesting that simvastatin might also inhibit the expression of these genes via the repressive activity of PPARα.

Similar to what was observed for the PPARγ agonist BRL49653, the IFN-γ-induced STAT1 phosphorylation was not affected by GW7647 (Figure 5.11). These data suggest that GW7647 exerts its transrepression activity through mechanisms different from modulation of STAT1 phosphorylation. In contrast to our findings in THP-1 macrophages, Jee et al (2005) showed that PPARα agonists inhibit the LPS-induced STAT1 phosphorylation and transactivation in rat brain glial cells. PPARα agonists thus probably exert their transrepression activity through mechanisms depending on the stimuli and the cell type.

5.7.4 Effects of LXR agonists on IFN-γ signalling

As shown in Figure 5.12 and Table 5.2, the synthetic LXR agonist TO901317 significantly attenuated the IFN-γ-induced expression of MCP-1 and CIITA pIV. Such slight but reproducible effects confirm the anti-inflammatory and immunomodulatory actions of LXR agonists as previously suggested by numerous studies [see (Pascual and Glass, 2006; Rizzo and Fiorucci, 2006; Bensinger and Tontonoz, 2008) for reviews]. Although the expression of these three genes in response to IFN-γ is known to be mediated by STAT1-dependent signalling, the IFN-γ-induced phosphorylation of this transcription factor was not affected by TO901317 (Figure 5.13), thereby suggesting the existence of a mechanism independent of STAT1 phosphorylation. Previous studies have proposed several mechanisms by which TO901317

transrepresses pro-inflammatory stimuli-induced gene expression, such as targeting of the NFκB and AP-1-dependent pathways (Castrillo et al., 2003; Ogawa et al., 2005; Piraino et al., 2006; Zhang-Gandhi and Drew, 2007). However, to our knowledge, there have been no published reports showing that the agonist represses inflammatory gene expression by targeting STAT1 activity.

In contrast to TO901317, the endogenous LXR ligand, 22R, and the RXR ligand, 9cRA, attenuated the IFN-γ-induced STAT1 phosphorylation in THP-1 macrophages individually or in combination (Figures 5.13B and 5.14). LXRs are known to form heterodimers with RXRs in order to bind to LXRE in the promoter of target genes to activate transcription (Aranda and Pascual, 2001). In addition, the heterodimer can be activated by ligands for both LXRs and RXRs, either separately or in synergy (Aranda and Pascual, 2001). Consistent with this, Figure 5.13B shows that 22R together with 9cRA inhibited the IFN-γ-induced STAT1 phosphorylation to a greater extent than the inhibitory effect of individual agonist. In addition to STAT1 phosphorylation, 22R and 9cRA inhibited the activation of STAT1 response elements and MCP-1 gene promoter in response to IFN-γ (Figure 5.16). To our knowledge, this is the first time that a ligand-dependent activation of LXR has been shown to repress the IFN-γ-induced gene expression by inhibiting STAT1 phosphorylation and transactivation.

In contrast to its essential role in the LXR-mediated activation of gene transcription, LXREs have not been identified in the proximal promoters of LXR-repressed inflammatory genes (Castrillo et al., 2003; Ogawa et al., 2005; Terasaka et al., 2005; Zelcer and Tontonoz, 2006). Moreover, as discussed in the General Introduction (section 1.8.2), ligand-dependent activation of LXRs is thought to suppress inflammatory gene expression by transrepression (Pascual and Glass, 2006). In contrast to transcriptional activation, which involves the binding of LXRs to specific LXREs in the promoter or enhancer regions of target genes, transrepression operates through a mechanism independent of LXRE binding. It remains unclear whether the repression of gene expression by LXR requires heterodimerisation with RXR (Glass

and Ogawa, 2006). Therefore, although 9cRA has been clearly shown to inhibit the IFN-γ-induced STAT1 phosphorylation individually or in combination with 22R, the exact role of RXR in the 22R/9cRA-mediated inhibition of the IFN-γ-induced STAT1 phosphorylation and transactivation remains poorly understood. LXR/RXR, PPAR/RXR and Farnesoid X receptor (FXR)/RXR are known as 'permissive heterodimer', as demonstrated by the finding that an RXR ligand, such as 9cRA stimulates the transcriptional activity of the heterodimers. In addition to PPARs and LXRs, FXRs are also recently emerging as anti-inflammatory NRs (Li et al., 2007; Wagner et al., 2008; Zhang et al., 2009). Therefore, in addition to its potential action on LXR/RXR, the 9cRA-mediated repression of IFN-γ-induced STAT1 phosphorylation could also be because of its action on FXR/RXR and PPAR/RXR. It should however be noted that such potential effect of 9cRA on other NR does not contradict a specific effect of LXRs on IFN-γ responses because the LXR-dependent effects have been further confirmed using the synthetic ligand TO901317 (Figures 5.12 and 6.12) and LXR expression plasmids (Figure 6.17A) (see Chapter 6 for details).

It is becoming increasingly clear that non-genomic actions by NR ligands are an important aspect in their regulation of gene expression. Non-genomic actions of NR ligands are the actions that do not initially have a direct effect on gene transcription, but rather often involve the rapid generation of intracellular second messengers through the activation of various signal transduction cascades. Some of the non-genomic actions of steroids are mediated by classical NRs, while the others are thought to be mediated by novel, non-classical membrane receptors, the biological properties of which remain to be thoroughly investigated [see (Losel and Wehling, 2003; Wehling and Losel, 2006) for reviews]. Work in our laboratory has recently suggested such non-genomic actions for 22R and 9cRA in THP-1 macrophages by demonstrating that these ligands together regulate ABCA1 and apoE expression in THP-1 macrophages through pathways involving Akt, PKC and JNK/SAPK (Huwait, 2008). Results presented in the previous chapter have demonstrated important roles

for MAPK (i.e. ERK and JNK) and PKC in the IFN-γ-regulated gene expression. Interestingly, Yao et al. (2006) have recently shown that ERK and p38 MAPK negatively regulate the IFN-γ-induced expression of the CIITA gene in macrophages. It is thus of interest to find out whether these LXR/RXR ligands negatively regulate the IFN-γ-induced gene expression in macrophages through non-genomic actions by activation of signal transduction cascades, such as ERK.

5.7.5 Effects of therapeutic agents on the IFN-γ-induced adhesion of monocytes to endothelial cells

PPAR and LXR ligands are known to exert their athero-protective roles largely through inhibition of the recruitment of leukocytes to the site of inflammation (Straus and Glass, 2007; Nomiyama and Bruemmer, 2008). Suppressive effects of PPAR ligands in adhesion of monocytes to endothelial cells, and the subsequent transmigration have been documented (Marx et al., 1999; Ahmed et al., 2006; Barish et al., 2008). Consistent with these previous findings, pre-treatment of HUVEC with the endogenous PPARγ ligand 15d-PGJ2 and the endogenous LXR/RXR ligands 22R and 9cRA attenuated the IFN-γ-induced adhesion of U937 monocytes to monolayers of HUVEC. In contrast to 15d-PGJ2, a synthetic PPARγ agonist BRL49653 did not inhibit, but instead induced such adhesion (Figure 5.17). This finding is consistent with a previous study (Rival et al., 2002), which demonstrated that BRL49653 increases the adhesion of U937 to the endothelial EA.Hy926 cell line, while another PPAR γ agonists 15d-PGJ2 decreased it. A similar phenomenon has been also observed with PPARα ligands. Fenofibrate has been shown to inhibit the adhesion of U937 to endothelial EA.Hy926 cells while another PPARα activator fenofibric acid induces it (Rival et al., 2002). These results suggest that distinct type of PPAR ligands might have different effect on cellular responses. However, the exact underlying reasons for such opposite effects of 15d-PGJ2 and BRL49653 in leukocyte adhesion to endothelial cells remain unclear at present.

It is worth noting that as the addition of 15d-PGJ2 or 22R together with 9cRA resulted in a low viability of HUVEC (Figure 5.17), we cannot rule out the possibility that the reduced adhesion may be due to decreased viability of HUVEC. Interestingly, although pre-treatment of HUVEC with BRL49653 resulted in viability that was comparable to the other ligands, the adhesion was increased. It is therefore difficult to come to any firm conclusions on whether the inhibition of adhesion was due to specific action of 15d-PGJ2 and 22R/9cRA or because of reduced HUVEC viability. As suggested in Chapter 4 (section 4.3.9), the low viability of HUVECs is probably due to the prolonged period of incubation (32h). Alternative approaches will therefore be required in the future for such studies. For example, Marx et al. (1999) have demonstrated that the TNF-α-induced adhesion of U937 monocytes to human saphenous vein endothelial cells (HSVEC) was inhibited by the PPARα agonist fenofibrate without an effect on viability and protein synthesis of these cells despite the incubation period of over 32h. Therefore, the use of HSVEC instead of HUVEC provides one potential approach.

CHAPTER SIX:

INVESTIGATION INTO MECHANISMS UNDERLYING THE INHIBITORY EFFECTS OF THE THERAPEUTIC AGENTS ON IFN-γ SIGNALLING

Chapter 6: Investigation of mechanisms underlying the inhibitory effects of therapeutic agents on IFN-γ signalling

6.1 Introduction

Work presented in Chapter 5 has shown that PPAR and LXR ligands exert significant inhibitory effects on the IFN-γ-induced expression of several genes in human macrophages, at least in part, by inhibition of STAT1-dependent transcriptional activation. As discussed in the General Introduction (section 1.8), although previous studies have proposed a number of trans-repression models for the actions of PPAR and LXR ligands, such as competition for a limiting amount of co-activators or prolonged recruitment of co-repressors, the exact mechanisms underlying the NR-dependent repression remain largely unclear, particularly in relation to IFN-γ actions and the JAK-STAT pathway.

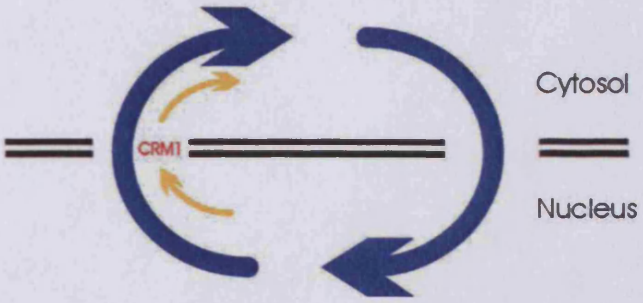
6.1.1 Nucleo-cytoplasmic translocation of STAT1

STAT proteins are latent transcription factors that mainly reside in the cytoplasm before activation. Although immunofluorescence microscopy shows a predominantly cytoplasmic localisation of most STATs in resting cells, it is well recognised that inactive STATs are constantly shuttling between the nucleus and the cytosol via a carrier-independent mechanism. In addition to this, the export receptor CRM1-dependent export also operates constitutively (Andrews et al., 2002; Zeng et al., 2002), although it only plays a minor role in the overall flux rate of STATs (Marg et al., 2004). Carrier-dependent nuclear import has so far not been reported for the inactive STAT1 protein (Figure 6.1A).

As detailed in the General Introduction (section 1.6), the binding of IFN-γ to its cognate receptors leads to the activation of JAK1 and JAK2, and the tyrosine phosphorylation of STAT1 (Tyr701). This process, commonly termed STAT1

activation triggers the dimerisation of STAT1 and, subsequently, fast and efficient translocation into the nucleus, where they bind to STAT1 response elements (e.g. GAS) and activate transcription of IFN-γ-inducible genes [see (Darnell, 1997; Stark et al., 1998) for reviews]. Dimerisation has been shown to reduce the carrier-free nuclear import of activated STAT1, and switch to carrier-dependent translocation (Sekimoto et al., 1997; Marg et al., 2004). STATs contain nuclear localisation signals (NLSs), which interact with importin α5. This then binds to importin β which docks the STAT complex to nuclear pores and initiate its translocation into the nucleus. Only STAT dimers that have two intact NLS elements, one from each monomer, are able to bind to importin α5 (Sekimoto et al., 1996; Sekimoto et al., 1997; Fagerlund et al., 2002). Nuclear import and retention are two separate steps leading up to nuclear accumulation, with non-specific DNA binding of activated STAT1 being sufficient for nuclear retention. Critical for nuclear accumulation of STAT1 and the subsequent nuclear export is the point of STAT1 tyrosine dephosphorylation, as activated STAT1 is incapable of leaving the nucleus (Meyer et al., 2003). It has been demonstrated that DNA binding protects STAT1 from dephosphorylation in a DNA sequence-specific manner (Meyer et al., 2003), and hence reveals a critical role for non-specific DNA binding in the nuclear retention of STAT1 (Vinkemeier, 2004) (Figure 6.1B).

A. Resting Cells



B. IFN-γ-stimulated Cells

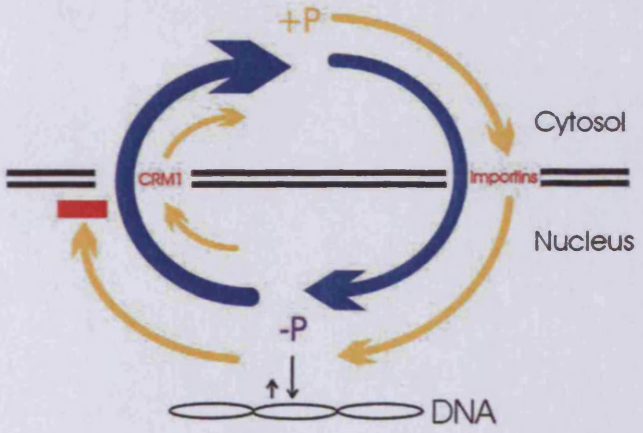


Figure 6.1 Schematic representation of STAT1 nucleo-cytoplasmic cycling in resting cells (A) and during IFN-γ stimulation (B) [Taken from (Meyer et al., 2003; Vinkemeier, 2004)]

Carrier-independent cycling is depicted with blue arrows and carrier-dependent translocation with yellow arrows. The red bar represents the export block of phosphorylated STAT1 dimers. Phosphorylation and dimerisation are indicated (+P). The reverse reaction (dephosphorylation) is indicated (-P). Arrow widths are proportional to their corresponding flux rate.

6.1.2 Co-activator competition transrepression model for activated NRs

NRs, such as PPARs and LXRs, and many of the transcription factors that drive inflammatory responses require an overlapping set of co-activator proteins (Huuskonen et al., 2004; Ricote and Glass, 2007). Previous studies have identified a large number of such co-regulators, including the p160 family proteins (such as steroid receptor co-activator-1 (SRC-1)) and the CBP/p300 (Lee et al., 2000; Huuskonen et al., 2004). Upon ligand binding, the activation function (AF)-2 subdomain of NRs undergo a conformation change, which allows the direct interaction with the p160 family co-activators (e.g. SRC-1). These co-activators can also interact with CBP/p300 proteins via a separate domain. Additionally, direct interaction between CBP/p300 with NRs has also been reported, which may enhance complex formation (Aranda and Pascual, 2001). Furthermore, SRC-1 has been reported to recruit additional co-factors, such as CARM1 and p300/CBP-associated factors (PCAF), to target gene promoters. Once these complexes have been assembled, the histone acetylase activities of CBP/p300 and PCAF, and the histone methyltransferase activity of co-activator-associated arginin methyltransferase-1 (CARM1) serve to remodel the chromatin architecture at target gene promoters, thus promoting transcriptional activation (Darimont et al., 1998; Leo and Chen, 2000). SRC-1 proteins have also been shown to be involved in transactivation by several transcription factors, including AP-1, NFκB, serum response factor (SRF) and p53 (Lee et al., 2001). CBP and p300 are evolutionary conserved proteins that serve as co-activators for different types of transcription factors, including AP-1, MyoD, NFκB, Pit-1 and STATs by potentiating their transcriptional activity (Shikama et al., 1998).

Several studies have shown that competition for a limiting amount of co-activators, such as SRC-1 and CBP/p300 in cells could, at least in part, be responsible for the antagonistic actions of NR ligands on activities of transcription factors. For example,

Li et al. (2000b) have shown a strong correlation between PPARγ interaction with co-activators and the repression of the iNOS promoter in response to ligand binding. This is the so called co-activator competition transrepression model for NRs (Pascual and Glass, 2006). As discussed in the General Introduction (section 1.8), PPARs and LXRs have been shown to repress IFN-γ responses by antagonising the actions of various signalling cascades, such as NFκB-, JAK/STAT1- and AP-1-dependent pathways. Noteworthily, Giri et al. (2004) have shown that the PPARγ agonist, 15d-PGJ2, inhibits the IFN-γ and LPS-induced NFκB-dependent pathway by reducing the recruitment of p300 to the promoter of target genes. It is thus of great interest to know whether the co-activator competition model is also used for the inhibitory actions of PPARs and LXRs on IFN-γ-stimulated gene transcription, especially through the classical JAK-STAT pathway.

6.1.3 Experimental strategy

Work in this chapter was carried out with the aim of investigating the potential mechanisms by which statins and PPAR and LXR ligands inhibit the IFN-γ-stimulated signalling pathways as detailed in the previous chapter. Simvastatin, PPARγ agonists (BRL49653 and 15d-PGJ2), PPARα agonists GW7647, synthetic LXR agonist TO901317 and the endogenous LXR/RXR ligands 22R and 9cRA were selected for further studies because they were previously shown to mediate the inhibition of IFN-γ-induced gene expression and cellular responses in human macrophages (Figures 5.2-7). Previous work presented in Chapter 5 has suggested that simvastatin, PPAR-α and -γ agonists and TO901317 exert their repressive activities on the IFN-γ-induced gene expression, at least in part, by inhibiting STAT1 transactivation but without affecting STAT1 phosphorylation (section 5.7). We therefore decided to investigate the potential mechanisms underlying such repressive activities with emphasis on STAT1-dependent pathways. First, we decided to investigate the effects of these therapeutic agents on nucleo-cytoplasmic

shuttling of activated STAT1 in response to IFN-γ. This was achieved by analysing the amounts of phospho-Tyr701 STAT1 in nuclear and cytoplasmic fractions using western blot analysis. In addition, the effect of these therapeutic agents on the IFN-γ-induced binding of STAT1 to GAS elements in the MCP-1 promoter was investigated using EMSA analysis.

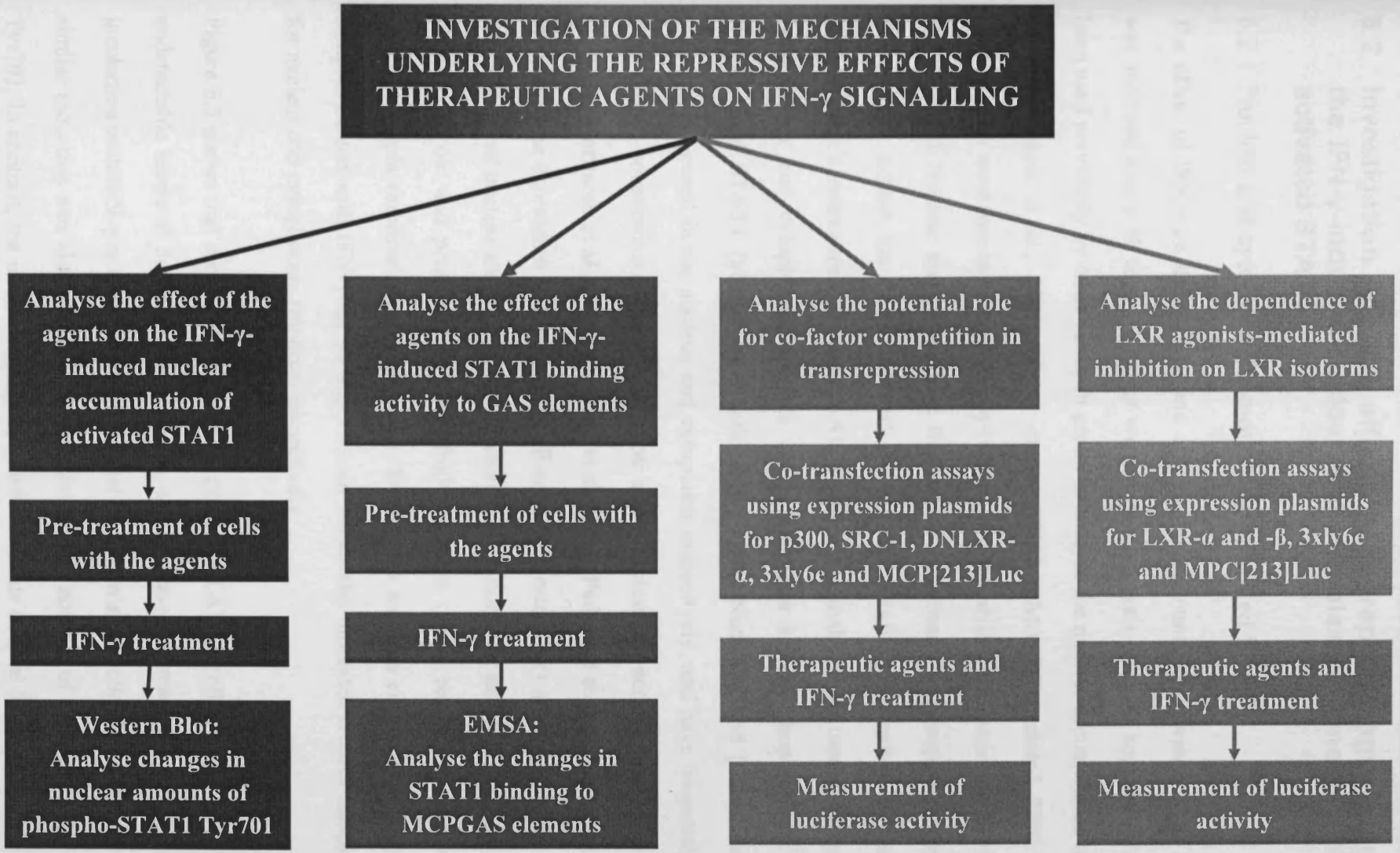
In order to investigate whether PPAR and LXR ligands repress the IFN-γ-induced STAT1 transcriptional activity by competing for limiting amounts of the co-activators p300 and SRC-1 in cells, we first decided to carry out co-transfection assays using the plasmid containing luciferase reporter gene driven by STAT1 response elements (3xly6e) and expression plasmids for p300 and SRC-1. Furthermore, the hypothesis that repression can be caused by competition among different transcription factors for limiting amounts of essential co-activators predicts that mutations affecting co-activator binding and/or transactivation should also affect repression (Li et al., 2000b). To test this hypothesis, we examined the effects of deletions in the AF-2 subdomain of LXR on the ligand-dependent repression of IFN-γ-mediated activation of STAT1 response elements and MCP-1 promoter by transient transfection assays. DNLXR-α, which contains the full length sequence of LXR-α lacking the AF-2 subdomain, was therefore employed in this study (Kocarek et al., 2002). AF-2 is known to be required for the recruitment of co-activators to NRs and serve as an adapter surface for interactions with other molecules necessary for transcriptional activation ((Barettino et al., 1994; Bourguet et al., 2000). Point mutations in the AF-2 domain has been shown to abolish transcriptional activation (Svensson et al., 2003).

Finally, as shown in the previous chapter (Figures 5.14-17), the LXR ligand, 22R and the RXR ligand, 9cRA, repress the IFN-γ-induced STAT1 phosphorylation and transactivation. In order to further investigate the roles of LXR-α and -β isoforms in the 22R and 9cRA-mediated repression, co-transfection assays were employed again

using the 3xly6e plasmid and expression vectors for LXR isoforms. Figure 6.2 summaries the overall experimental approach.

Figure 6.2 Experimental Strategy

The aim of the experimental strategy was to investigate the potential mechanisms underlying the therapeutic agents (statins, agonists of PPAR-α and -γ and LXRs)-mediated inhibition of IFN-γ signalling pathways. This was achieved by analysis of the effects of these agents on the IFN-γ-induced nuclear accumulation of phospho-STAT1 Tyr701 and STAT1 binding to MCPGAS elements. In addition, for analysis of whether agonists for PPARs and LXRs repress IFN-γ signalling by competition with transcription factors for limiting amounts of co-activators, transient transfection assays were performed using expression plasmids for p300 and SRC-1 and a DN plasmid for LXR-α lacking the AF-2 subdomain (DNLXR-α). Dependence of LXR agonists-mediated inhibition on LXR isoforms was also investigated by transient transfection assays using expression plasmids for LXR-α and -β.


INVESTIGATION OF THE MECHANISMS UNDERLYING THE REPRESSIVE EFFECTS OF THERAPEUTIC AGENTS ON IFN-γ SIGNALLING
Analyse the effect of the agents on the IFN-γ-induced nuclear accumulation of activated STAT1
Pre-treatment of cells with the agents
IFN-γ treatment
Western Blot: Analyse changes in nuclear amounts of phospho-STAT1 Tyr701
Analyse the effect of the agents on the IFN-γ-induced STAT1 binding activity to GAS elements
Pre-treatment of cells with the agents
IFN-γ treatment
EMSA: Analyse the changes in STAT1 binding to MCPGAS elements
Analyse the potential role for co-factor competition in transrepression
Co-transfection assays using expression plasmids for p300, SRC-1, DNLXR-α, 3xly6e and MCP[213]Luc
Therapeutic agents and IFN-γ treatment
Measurement of luciferase activity
Analyse the dependence of LXR agonists-mediated inhibition on LXR isoforms
Co-transfection assays using expression plasmids for LXR-α and -β, 3xly6e and MPC[213]Luc
Therapeutic agents and IFN-γ treatment
Measurement of luciferase activity

6.2 Investigation into the effects of therapeutic agents on the IFN-γ-induced nuclear and cytoplasmic amounts of activated STAT1

6.2.1 Nuclear and cytoplasmic amounts of activated STAT1

The effect of IFN-γ on the nuclear and cytoplasmic amounts of activated STAT1 was analysed over a 3h time course by western blot analysis. Such an approach has been used previously by other research group to analyse the nuclear accumulation of STAT1 (Haspel et al., 1996; Meyer et al., 2002). PMA-differentiated THP-1 macrophages were incubated with IFN-γ (1000U/ml) for periods of 30min and 3h or left untreated. Nuclear and cytoplasmic fractions were subsequently prepared using the nuclear extract kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions (section 2.6.4). Western blot analysis was carried out on both nuclear and cytoplasmic extracts using antibodies against phopho-STAT1 Tyr701, total STAT1 (p84/91), nucleolin and β-actin. Nucleolin and β-actin are primarily presented in the nucleus and cytoplasm respectively, and have frequently been used as constitutive controls for nuclear and cytoplasmic fractions (McHugh et al., 1991; Morimoto et al., 2002; Akiyama et al., 2003; Padilla et al., 2004). We thus monitored the expression of nucleolin and β-actin by western blot analysis to ensure the integrity of nuclear and cytoplasmic fractions. Densitometric analysis of the data was carried out and presented as mean fold induction (±SD) relative to nuclear sample at 0min (arbitrarily assigned as 1). Student's *t* test was carried out on the samples treated with IFN-γ for 30 and 180min versus the untreated control sample for nuclear and cytoplasmic fractions respectively.

Figure 6.3 shows that the nuclear amount of phopho-STAT1 Tyr701 was at almost undetectable levels in the 0min control cells, and increased dramatically following incubation with IFN-γ to reach the highest level at 30min and declined at 180min. A similar induction was also observed in the cytoplasmic amount of phospho-STAT1 Tyr701. In contrast, the nuclear and cytoplasmic amounts of total STAT1 remained

unchanged throughout the time-course. The induction of phospho-STAT1 Tyr701 and the unchanged amount of total STAT1 p84/91 was consistent with previous findings using whole cell extracts (Figure 3.11). These results suggest that IFN-γ rapidly induces the amount of phospho-STAT1 Tyr701 in the nucleus and the cytosol. It is worth noting that the expression of nucleolin and β-actin was predominantly detected in the nuclear and cytoplasmic fractions respectively, thereby demonstrating the intergity of nuclear and cytoplasmic fractions.

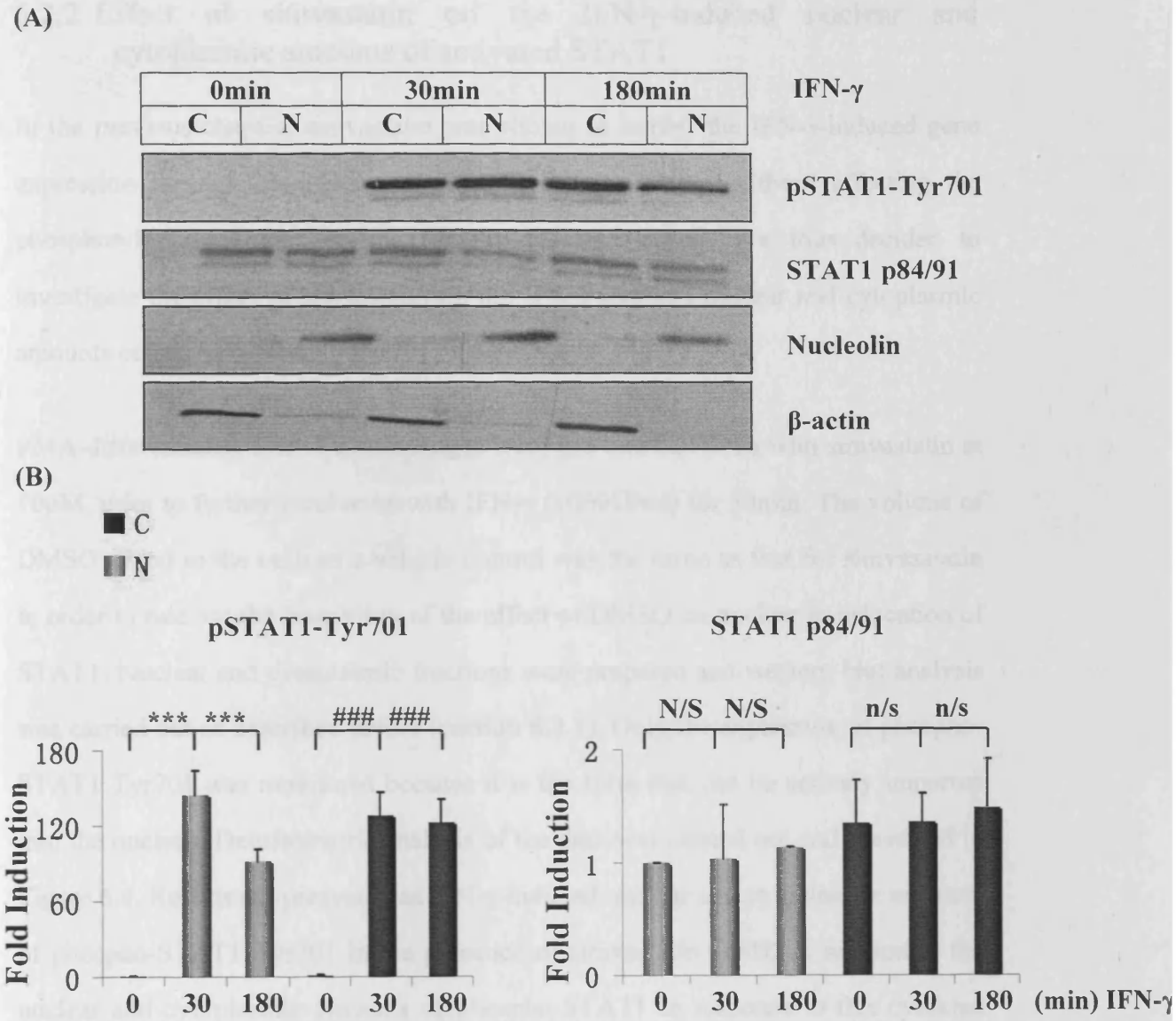


Figure 6.3 Effect of IFN-γ on nuclear and cytoplasmic amounts of STAT1 over time

PMA-differentiated THP-1 cells were treated with IFN-γ for the indicated period of time and nuclear (N), and cytoplasmic (C) extracts were prepared (section 2.6.4). Western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, STAT1 p84/91, nucleolin and β-actin (A). Panel B shows the fold induction (mean ±SD) of the levels of phospho-STAT1 Tyr701 and STAT1 p84/91 proteins relative to the 0h control (assigned as 1) from three independent experimental series. Student's *t* test was carried out on samples treated with IFN-γ for 30 and 180min versus 0h control sample for nuclear and cytoplasmic fractions, respectively (nuclear fraction: ***P<0.001, N/S not significant; cytoplasmic fraction: ###P<0.001; n/s not significant).

6.2.2 Effect of simvastatin on the IFN-γ-induced nuclear and cytoplasmic amounts of activated STAT1

In the previous chapter, simvastatin was shown to inhibit the IFN-γ-induced gene expression through modulation of STAT1 transactivation without affecting the phosphorylation of the protein (Figures 5.2 and 5.5-6). We thus decided to investigate the effect of simvastatin on the IFN-γ-induced nuclear and cytoplasmic amounts of phospho-STAT1 Tyr701.

PMA-differentiated THP-1 macrophages were pre-treated for 1h with simvastatin at 10μM, prior to further incubation with IFN-γ (1000U/ml) for 30min. The volume of DMSO added to the cells as a vehicle control was the same as that for simvasatatin in order to rule out the possibility of the effect of DMSO on nuclear translocation of STAT1. Nuclear and cytoplasmic fractions were prepared and western blot analysis was carried out as described before (section 6.2.1). Only the expression of phospho-STAT1 Tyr701 was monitored because it is the form that can be actively imported into the nucleus. Densitometric analysis of the data was carried out and presented in Figure 6.4. Results are presented as IFN-γ-induced nuclear and cytoplasmic amounts of phospho-STAT1 Tyr701 in the presence of simvastatin (±SD) in relation to the nuclear and cytoplasmic amounts of phospho-STAT1 in response to this cytokine respectively. The value from DMSO-treated sample stimulated with IFN-γ was arbitrarily assigned as 1. Student's *t* test was carried out on the simvastatin and cytokine-treated sample versus IFN-γ-induced level of phospho-STAT1 in nucleus and cytoplasm respectively.

Figure 6.4 clearly shows that pre-treatment of the cells with simvastatin had no effect on either the nuclear or cytoplasmic amounts of phospho-STAT1 Tyr701 in the presence of IFN-γ.

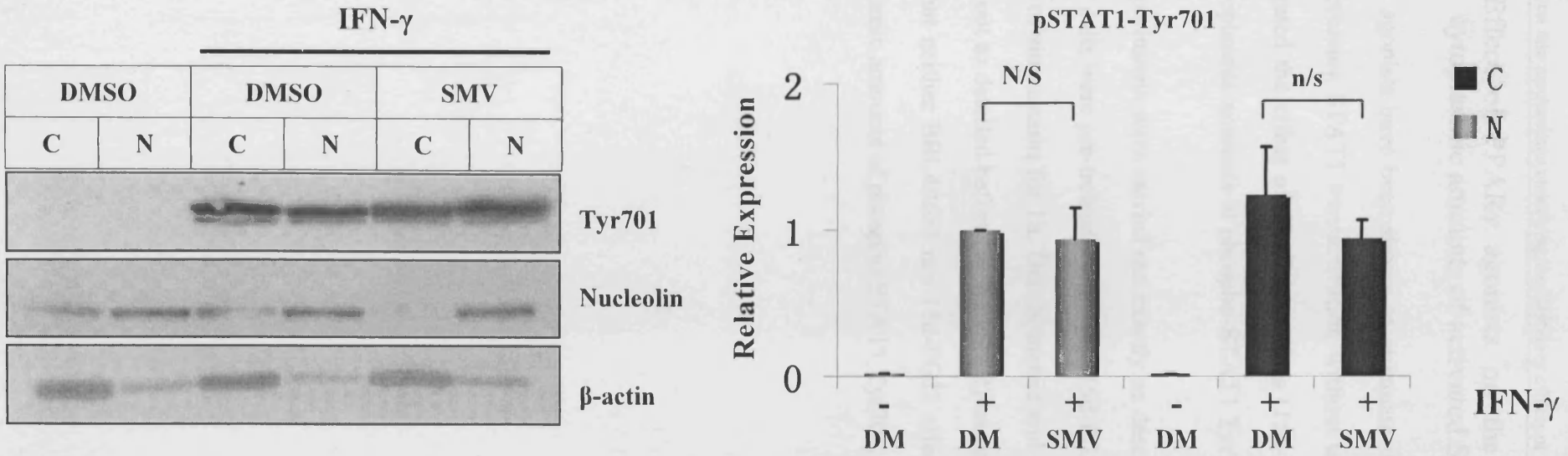


Figure 6.4 Effect of simvastatin on the amount of phospho-STAT1 in nucleus and cytoplasm

PMA-differentiated THP-1 cells were pre-treated for 1h with simvastatin (10μM) prior to further treatment with IFN-γ for 30min. DMSO (DM) was used as a vehicle control. Nuclear (N) and cytoplasmic (C) extracts were prepared using Nuclear Extract Kit (section 2.6.4). Western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, nucleolin and β-actin (A). Panel B shows the relative expression (mean ±SD) of phospho-STAT1 Tyr701 in both nuclear and cytoplasmic extracts from three independent experimental series (the value from nuclear extracts for IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Student's *t* test was carried out on nuclear and cytoplasmic fractions of samples treated with simvastatin versus nuclear and cytoplasmic fractions respectively of IFN-γ-stimulated DMSO control sample (nuclear fraction: N/S not significant; cytoplasmic fraction: n/s not significant).

6.2.3 Effect of PPARγ agonists on the IFN-γ-induced nuclear and cytoplasmic amounts of activated STAT1

PPARγ agonists have been shown to attenuate the IFN-γ-induced gene expression by suppressing STAT1 transactivation without targeting phosphorylation. We thus investigated the effect of PPARγ agonists (15d-PGJ2 and BRL49653) on nuclear and cytoplasmic amounts of phospho-STAT1 Tyr701.

The experiments were carried out exactly as described before (section 6.2.2) except that the cells were pre-treated for 2h with 15d-PGJ2 (10μM) and BRL49653 (20μM) instead of simvastatin for 1h. Densitometric and statistical analysis of the data was carried out as detailed before (section 6.2.2) and presented in Figure 6.5. The results show that neither BRL49653 nor 15d-PGJ2 affected the IFN-γ-induced nuclear or cytoplasmic amounts of phospho-STAT1 Tyr701.

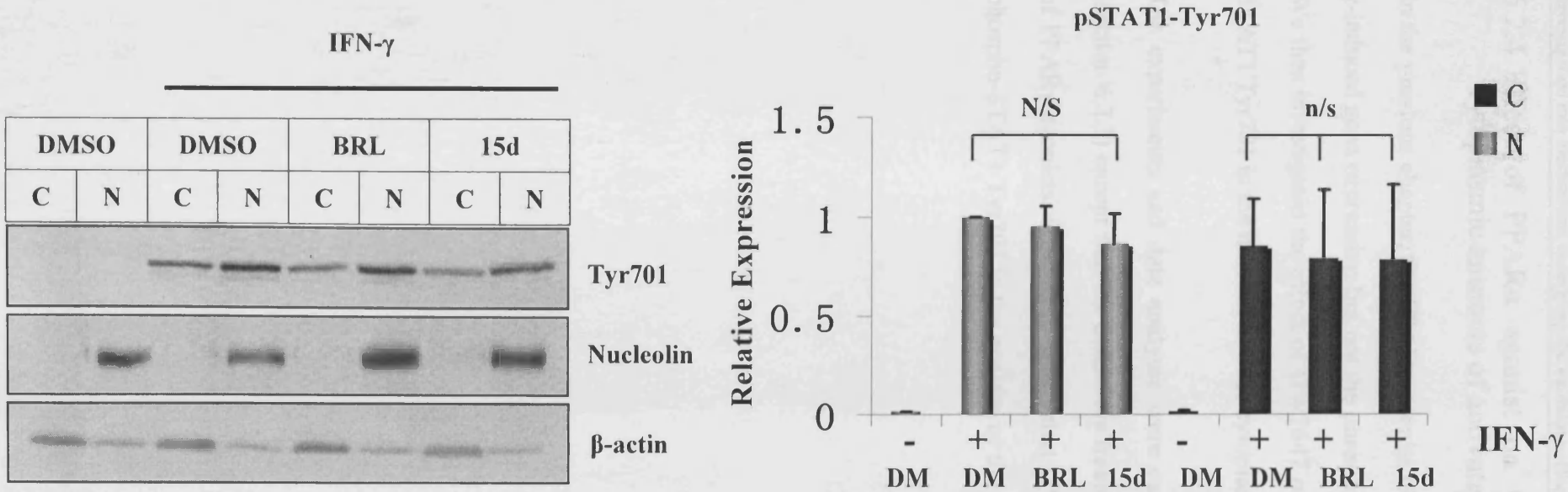


Figure 6.5 Effect of PPARγ agonist on the IFN-γ-induced amount of phospho-STAT1 in the nucleus and the cytoplasm

PMA-differentiated THP-1 cells were pre-treated for 2h with BRL49653 (BRL, 20μM) or 15d-PGJ2 (15d, 10μM) prior to further treatment with IFN-γ for 30min. DMSO (DM) was used as a vehicle control. Nuclear (N) and cytoplasmic (C) extracts were prepared using the Nuclear Extract Kit (section 2.6.4). Western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, nucleolin and β-actin (A). Panel B shows the relative expression (mean ±SD) of phospho-STAT1 Tyr701 in both nuclear and cytoplasmic extracts from three independent experimental series (the value from nuclear extract of IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Student's *t* test was carried out on nuclear and cytoplasmic extract samples treated with PPAR-γ agonist versus IFN-γ-stimulated DMSO control sample (nuclear fraction: N/S not significant; cytoplasmic fraction: n/s not significant).

6.2.4 Effect of PPARα agonist on the IFN-γ-induced nuclear and cytoplasmic amounts of activated STAT1

In the previous chapter, the PPARα agonist GW7647 was shown to inhibit the IFN-γ-induced gene expression but not the phosphorylation of STAT1 (Figures 5.11-12). We thus investigated the effect of GW7647 on the IFN-γ-induced level of phospho-STAT1 Tyr701 in the nucleus and the cytoplasm.

The experiments and data analyses were carried out exactly as described before (section 6.2.3) except that the cells were treated for 2h with GW7657 (5μM) instead of PPARγ agonists. Figure 6.6 shows that GW7647 failed to affect the amounts of phospho-STAT1 Tyr701 in the nucleus or the cytoplasm in response to IFN-γ.

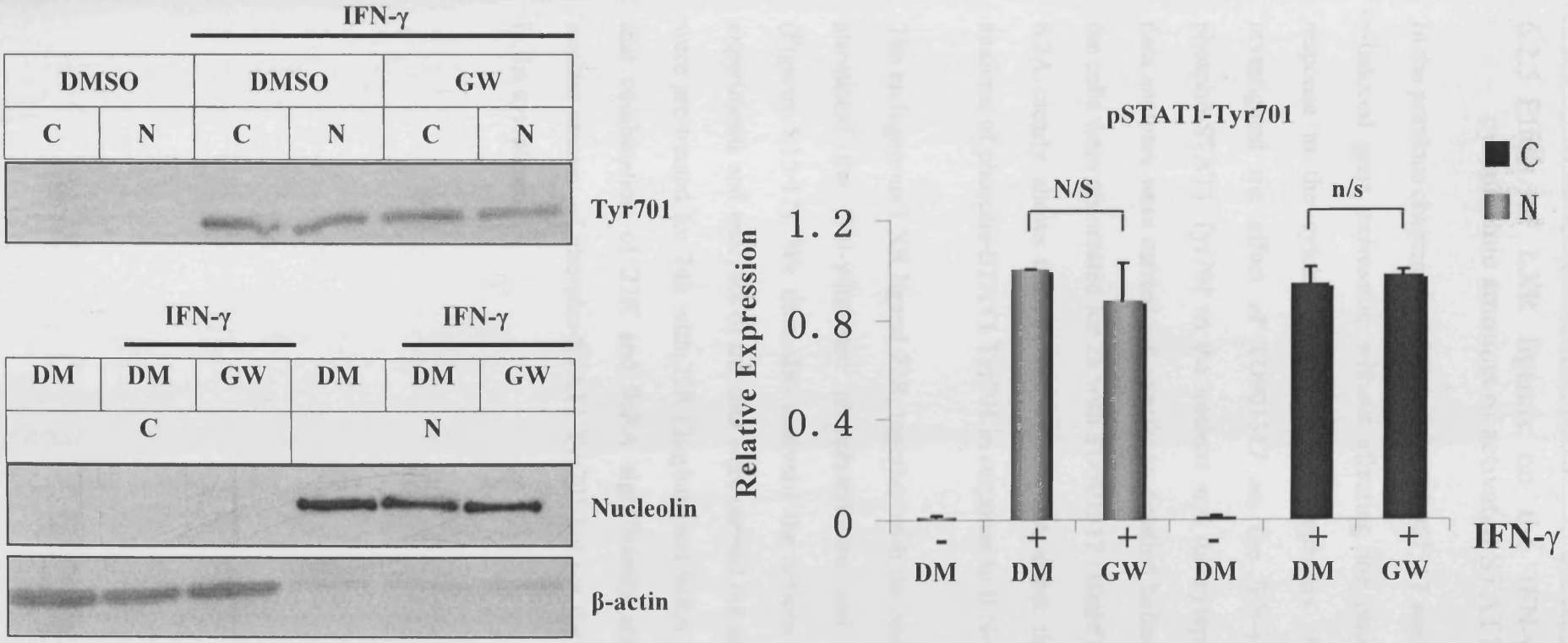
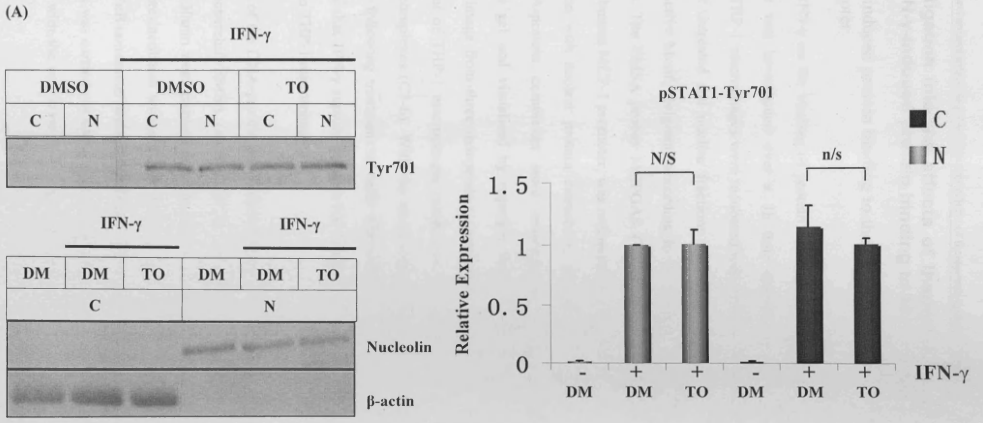


Figure 6.6 Effect of PPARα agonists on the IFN-γ-induced amount of phospho-STAT1 in the nucleus and the cytoplasm

PMA-differentiated THP-1 cells were pre-treated for 2h with GW7647 (5μM) prior to further treatment with IFN-γ for 30min. DMSO (DM) was used as a vehicle control. Nuclear (N) and cytoplasmic (C) extracts were prepared (section 2.6.4) and western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, nucleolin and β-actin (A). Panel B shows the relative expression (mean ±SD) of phospho-STAT1 Tyr701 in both nuclear and cytoplasmic extracts from three independent experimental series (the value from nuclear extract of IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Student's *t* test was carried out on nuclear and cytoplasmic extract samples treated with GW7647 versus IFN-γ-stimulated DMSO control sample (nuclear fraction: N/S not significant; cytoplasmic fraction: n/s not significant).



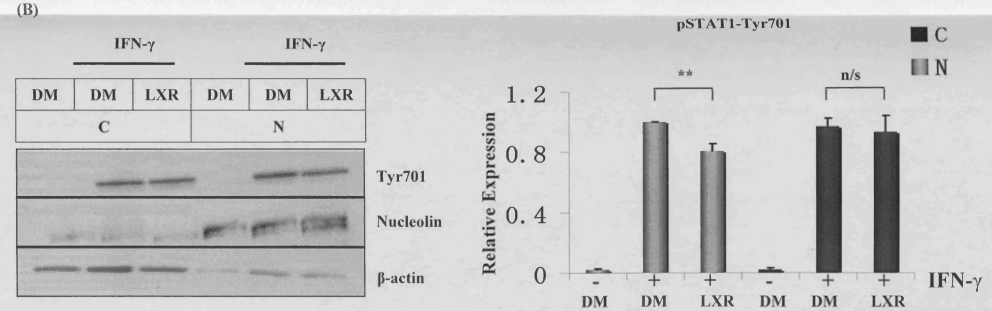


Figure 6.7 Effect of LXR agonists on the IFN-γ-induced amount of phospho-STAT1 in nucleus and cytoplasm

PMA-differentiated THP-1 cells were pre-treated for 2h with TO901317 (TO, 10μM) (A), or for 24h with the endogenous LXR ligands (LXR) 22R (2μg/ml) and 9cRA (10μM) prior to further treatment with IFN-γ for 30min. DMSO (DM) was used as a vehicle control. Nuclear (N) and cytoplasmic (C) extracts were prepared (section 2.6.4). Western blot analysis was carried out using antibodies specific for pSTAT1 Tyr701, nucleolin and β-actin (A). Panel B shows the relative expression (mean ±SD) of phospho-STAT1 at Tyr701 in both nuclear and cytoplasmic extracts from three independent experimental series (the value from nuclear extract of IFN-γ-treated DMSO control has been arbitrarily assigned as 1). Student's *t* test was carried out on nuclear and cytoplasmic extract samples treated with LXR agonists versus IFN-γ-stimulated DMSO control sample (nuclear fraction: $^{**}P<0.001$; cytoplasmic fraction: n/s, not significant).

6.3 Investigation into the effects of therapeutic agents on the IFN-γ-induced protein binding to MCP-1 promoter

6.3.1 IFN-γ induced protein binding to the GAS element on the MCP-1 promoter

The effect of IFN-γ on the binding of protein to the GAS element on the MCP-1 gene promoter was investigated over a 3h time course using EMSA. PMA-differentiated THP-1 macrophages were incubated with IFN-γ for periods of 30 and 180min or left untreated and nuclear fractions were prepared using the Nuclear Extract Kit (Active Motif, Belgium) according to the manufacturer's instructions (section 2.6.4). The EMSA probes MCPGAS (Table 2.10), containing the GAS element of the human MCP-1 promoter, was radiolabelled and incubated in a protein binding reaction with nuclear protein extracts as described in section 2.7. The resulting DNA-protein complexes were resolved on a 4% non-denaturing polyacrylamide gel and visualised by exposure to X-ray film (section 2.7). A representative image from three independent experiments is shown in Figure 6.8. IFN-γ treatment of THP-1 macrophages resulted in the formation of at least six DNA-protein complexes (C1-6). With the exception of C4, they were induced at varying extent following treatment of cells with IFN-γ for 30min and 3h. These results indicate that IFN-γ rapidly induces the binding of proteins to the MCP-1 GAS element in THP-1 macrophages.

The specificity of the DNA-protein interactions was next examined by competition- and antibody/supershift-EMSA (section 2.7.4). The nuclear extracts from IFN-γ treated cells at 30min were employed for the analysis. For competition-EMSA, the extracts were pre-incubated with a 250-fold molar excess of unlabelled MCPGAS, STAT1 and NFκB consensus binding site probes (Table 2.10). Antibody supershift-EMSA analysis was carried out using a STAT1 specific antibody (Santa Cruz, CA). Pre-incubation with the non-immune serum (NIS) served as a control. Subsequent

EMSA was carried out exactly as described previously. The representative image from three experiments is shown in Figure 6.8.

Protein binding of all complexes (C1-6) was competed out completely by an excess of the unlabelled MCPGAS probe and to a lesser extent by the unlabelled STAT1 consensus probe, but not by a consensus sequence for NFκB (Table 2.10). The competition analysis suggests that the formation of all complexes (C1-6) is specific to MCPGAS element and STAT1 protein was necessary for the formation of these complexes. Moreover, pre-incubation of the extracts with the anti-STAT1 antibody resulted in the formation of a slower migrating antibody-protein-DNA supershift complex (denoted ssSTAT1 in Figure 6.8). Formation of complexes C1-2, but not C3-6 was inhibited in the presence of the anti-STAT1 antibody, thereby indicating that C1-2 were likely to represent STAT1-DNA complexes. C1 and C2 probably represent DNA binding by STAT1 splice variant-α (p91) and -β (p84) respectively. On the other hand, pre-incubation of the extracts with the NIS did not affect the formation of any complexes (C1-6), thereby suggesting that the formation of antibody-protein-DNA supershift complex (ssSTAT1) is STAT1 specific. Together, these data suggest that STAT1, in nuclear protein extracts from IFN-γ-treated THP-1 macrophages, does specifically bind to the MCPGAS probe. As the binding of STAT1 (C1-2) to MCPGAS reached the highest level at 30min and declined at 180min, the 30min time point was selected for future EMSA.

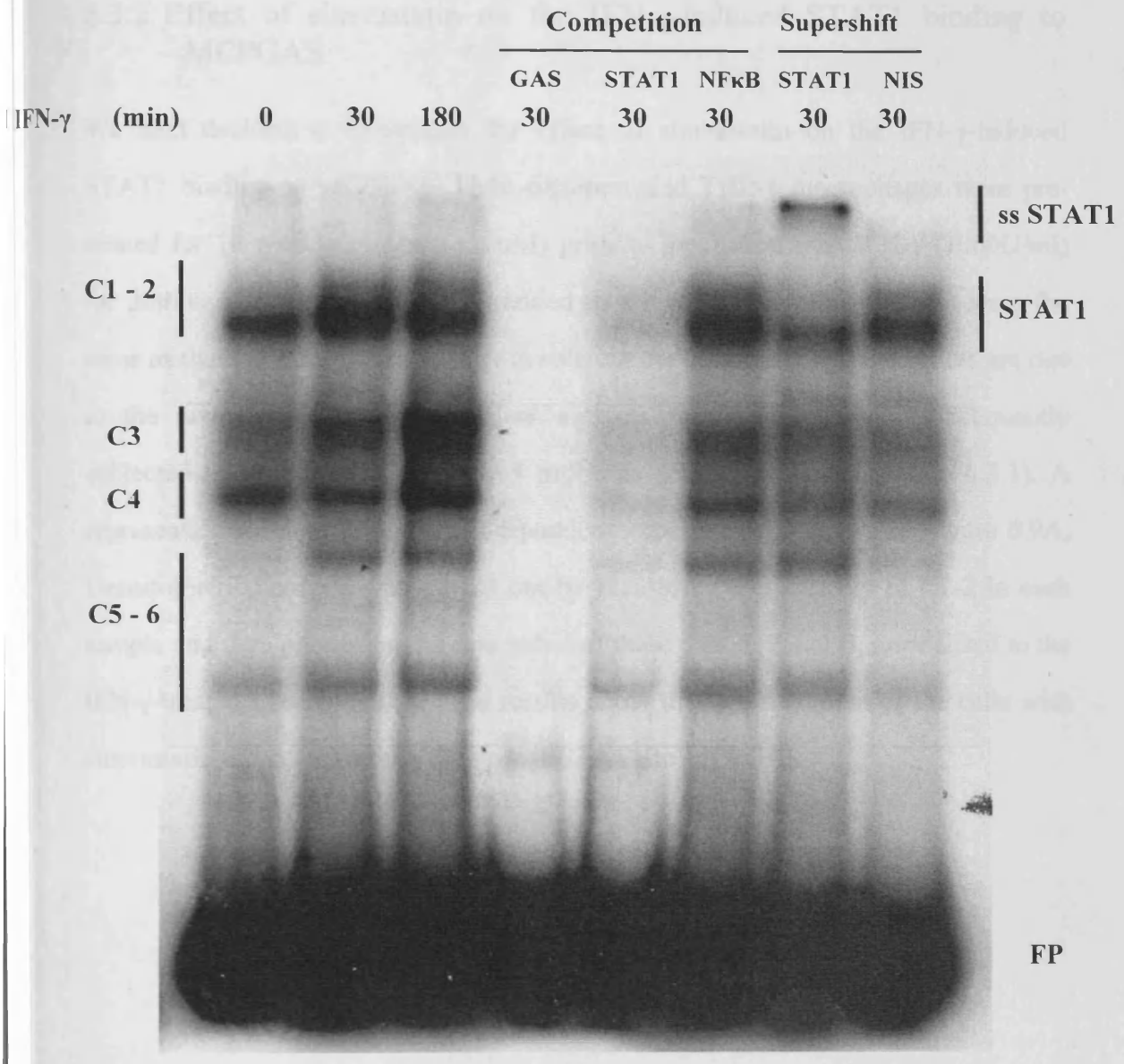


Figure 6.8 IFN-γ-induced formation of MCPGAS-protein complexes

PMA-differentiated THP-1 macrophages were treated with IFN-γ for 0, 30 and 180min and nuclear extracts were prepared. EMSA was carried out using the radiolabelled probe MCPGAS. The nuclear extracts prepared from the IFN-γ-treated cells (30min) were employed for competition and supershift EMSA. Competition assays were carried out using a 250x molar excess of unlabelled GAS, STAT1 and NFκB consensus sequences. Antibody supershift-EMSA was carried out using STAT1 specific antibody (Santa Cruz, CA). Pre-incubation with non-immune serum (NIS) served as a negative control. Results are representative of three independent experiments. ss denotes supershift; FP denotes free probes

6.3.2 Effect of simvastatin on the IFN-γ-induced STAT1 binding to MCPGAS

We next decided to investigate the effect of simvastatin on the IFN-γ-induced STAT1 binding to MCPGAS. PMA-differentiated THP-1 macrophages were pre-treated for 1h with simvastatin (10μM) prior to incubation with IFN-γ (1000U/ml) for 30min. The volume of DMSO added to the cells as a vehicle conrol were the same as that for simvastatin in order to rule out the possibility that the results are due to the presence of DMSO. Nuclear extracts were prepared and subsequently subjected to EMSA using MCPGAS probe as described before (section 6.3.1). A representative image from three independent experiments is shown in Figure 6.9A. Densitometric analysis was carried out by measuring the intensity of C1-2 in each sample and then presenting the sum value of these data in Panel B, normalised to the IFN-γ-treated DMSO control. The results show that pre-treatment of the cells with simvastatin did not affect the binding of STAT1 to MCPGAS.

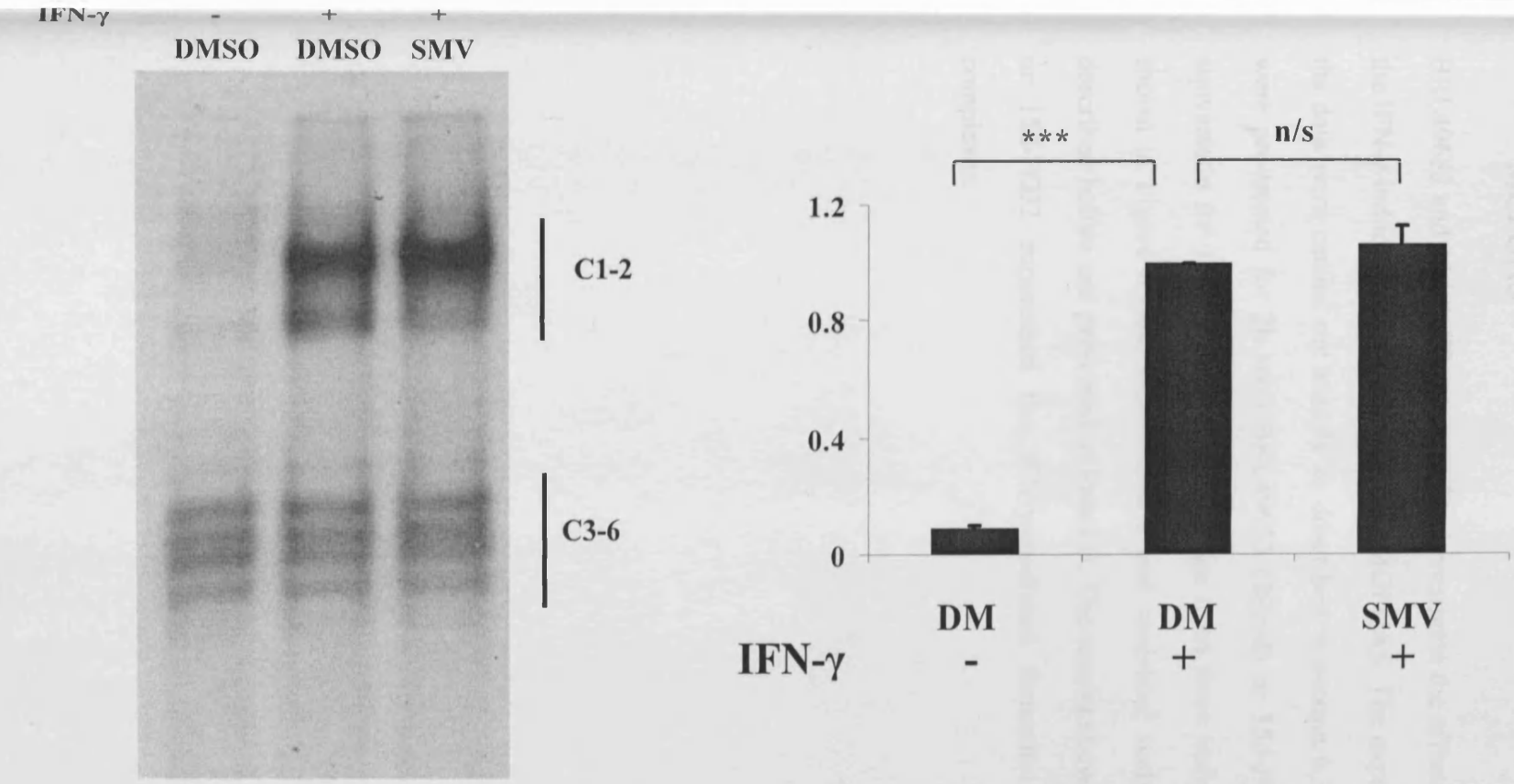


Figure 6.9 Effect of simvastatin on the IFN-γ-induced STAT1 binding to MCPGAS

PMA-differentiated THP-1 macrophages were pre-treated for 1h with simvastatin (10μM) or DMSO (DM) as a vehicle-control. They were then either stimulated with IFN-γ for 30min or left untreated. Nuclear extracts were prepared as before and used for EMSA using the radiolabelled MCPGAS consensus probe (A). The free probe has been run out of the gel. Densitometric analysis (determined by measuring the intensity of C1-2 in each sample and then calculating the sum value) is presented in Panel B, normalised to the IFN-γ-treated DMSO control. The results shown in Panel B are the mean ratio ± SD from three independent experimental series (***$P<0.001$, compared to the DMSO-treated control. n/s compared to the IFN-γ-treated DMSO control).

6.3.3 Effect of PPARγ agonists on the IFN-γ-induced STAT1 binding to MCPGAS

BRL49653 and 15d-PGJ2 were used to investigate the effects of PPARγ agonists on the IFN-γ-induced binding of STAT1 to MCPGAS. The experiments and analysis of the data were carried out exactly as described in section 6.3.2 except that the cells were pre-treated for 2h with BRL49653 (20μM) or 15d-PGJ2 (10μM) instead of simvastatin for 1h. A representative image from three independent experiments is shown in Figure 6.10A. Densitometric and statistical analysis was carried out as described before and presented in Panel B. The results show that neither BRL49653 or 15d-PGJ2 suppressed the IFN-γ-mediated formation of STAT1-MCPGAS complexes.

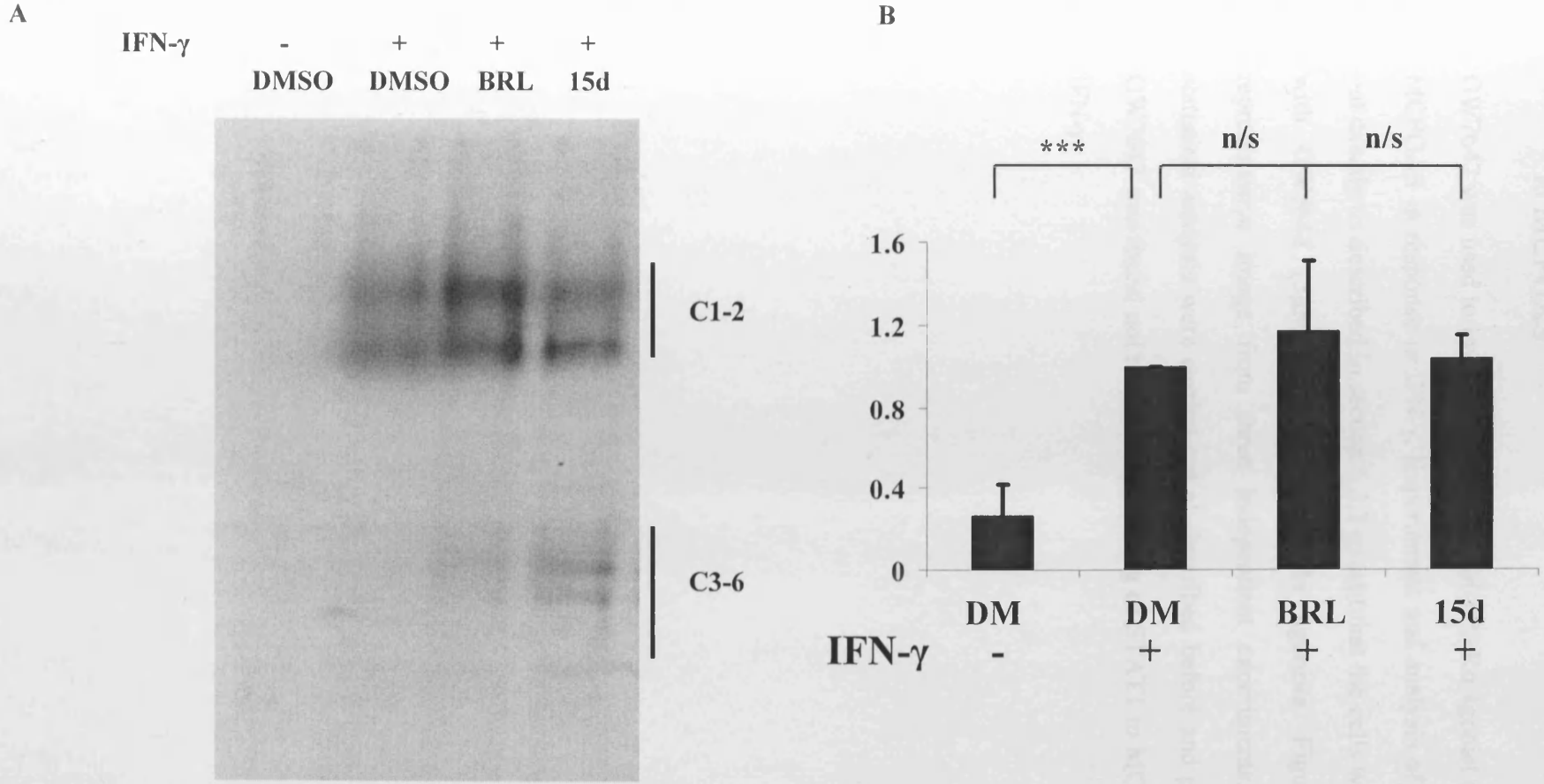


Figure 6.10 Effect of PPARγ agonists on the IFN-γ-induced STAT1 binding to MCPGAS

PMA-differentiated THP-1 macrophages were pre-treated for 2h with the BRL49653 (BRL, 20μM), 15d-PGJ2 (15d, 10μM) or DMSO (DM) as a vehicle-control. They were then either stimulated with IFN-γ for 30min or left untreated. Nuclear extracts were prepared as before and used for EMSA analysis using the radiolabelled MCPGAS consensus probe (A). The free probe has been run out of the gel. Densitometric analysis (determined by measuring the intensity of C1-2 in each sample and then calculating the sum value) of these data is presented in Panel B, normalised to the IFN-γ-treated DMSO control. The results shown in Panel B are the mean ratio ± SD from three independent experimental series (***$P<0.001$, compared to the DMSO-treated control; n/s compared to the IFN-γ-treated DMSO control).

6.3.4 Effect of PPARα agonists on the IFN-γ-induced STAT1 binding to MCPGAS

GW7647 was used to investigate the effect of PPARα agonist on STAT1 binding to MCPGAS in response to IFN-γ. Experiments and analysis of the data was carried out exactly as described in section 6.3.3 except that the cells were pre-treated for 2h with GW7647 (5μM) instead of PPARγ agonists. Figure 6.11A shows a representative image from three independent experiments. Densitometric and statistical analysis were carried out as described before and presented in Panel B. GW7647 was found not to affect the binding of STAT1 to MCPGAS in response to IFN-γ.

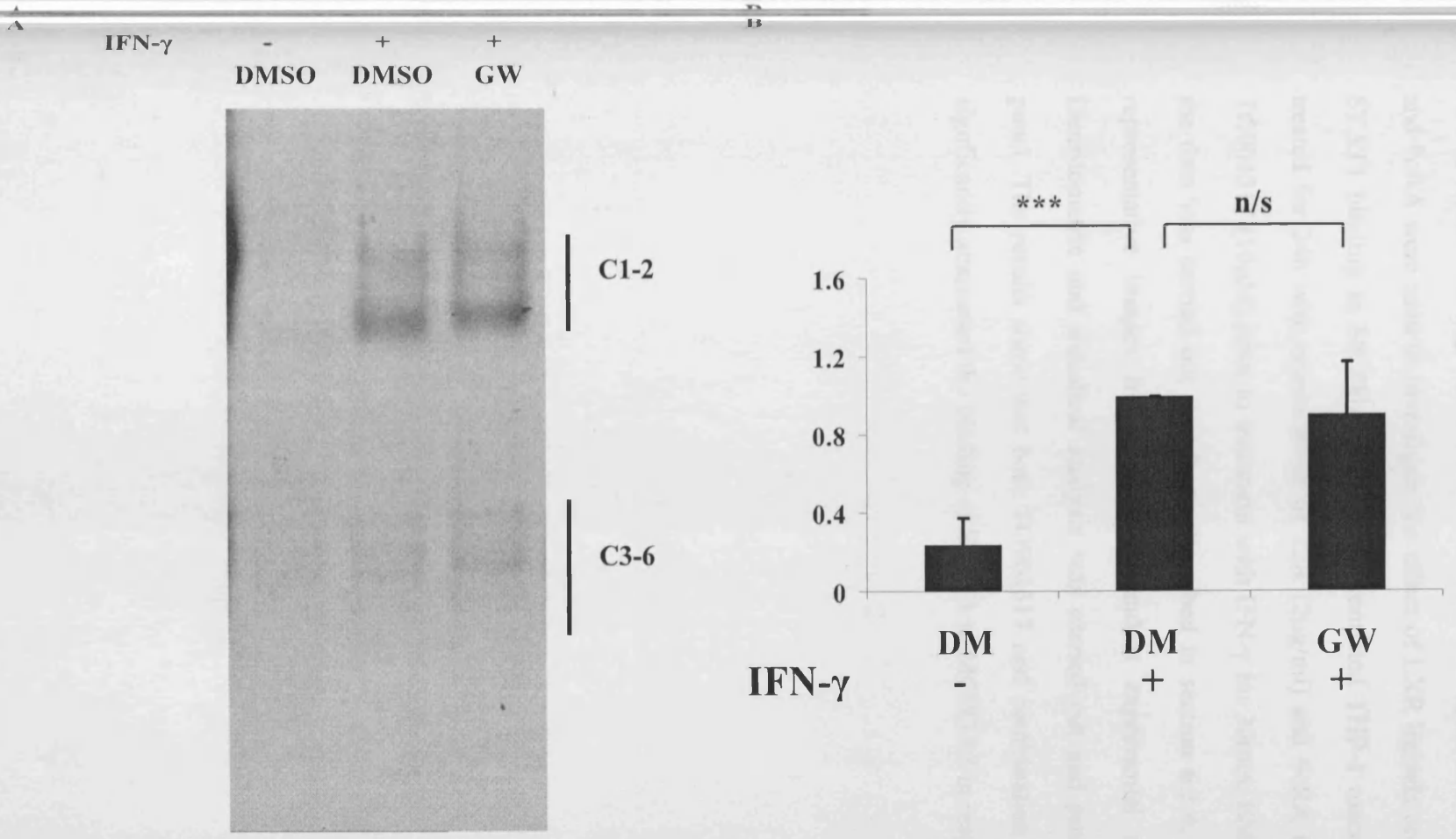


Figure 6.11 Effect of PPARα agonists on the IFN-γ-induced STAT1 binding to MCPGAS

PMA-differentiated THP-1 macrophages were pre-treated for 2h with the GW7647 (GW, 5μM) or DMSO (DM) as a vehicle-control. They were then either stimulated with IFN-γ for 30min or left untreated. Nuclear fractions were prepared as before and used for EMSA analysis using the radiolabelled MCPGAS probe (A). The free probe has been run out of the gel. Densitometric analysis (determined by measuring the intensity of C1-2 in each sample and then calculating the sum value) of these data is presented in Panel B, normalised to the IFN-γ-treated DMSO control. The results shown in Panel B are the mean ratio ± SD from three independent experimental series (***P<0.001, compared to the DMSO-treated control. n/s compared to the IFN-γ-treated DMSO control).

6.3.5 Effect of LXR agonists on the IFN-γ-induced STAT1 binding to MCPGAS

The synthetic LXR agonist TO901317 and the endogenous LXR/RXR ligands 22R and 9cRA were used to investigate the effect of LXR ligands on the IFN-γ-induced STAT1 binding to MCPGAS. PMA-differentiated THP-1 macrophages were pre-treated for 24h with combination of 22R (2μg/ml) and 9cRA (10μM) or 2h with TO901317 (10μM) prior to treatment with IFN-γ for 30min. EMSA and analysis of the data was carried out exactly as described in section 6.3.4. Figure 6.12 shows representative images from three independent exprimental series (left panel). Densitometric and statistical analysis was carried out and presented in the right panel. The results show that both TO901317 and combination of 22R and 9cRA signficantly attenuated the binding of STAT1 to MCPGAS in response to IFN-γ.

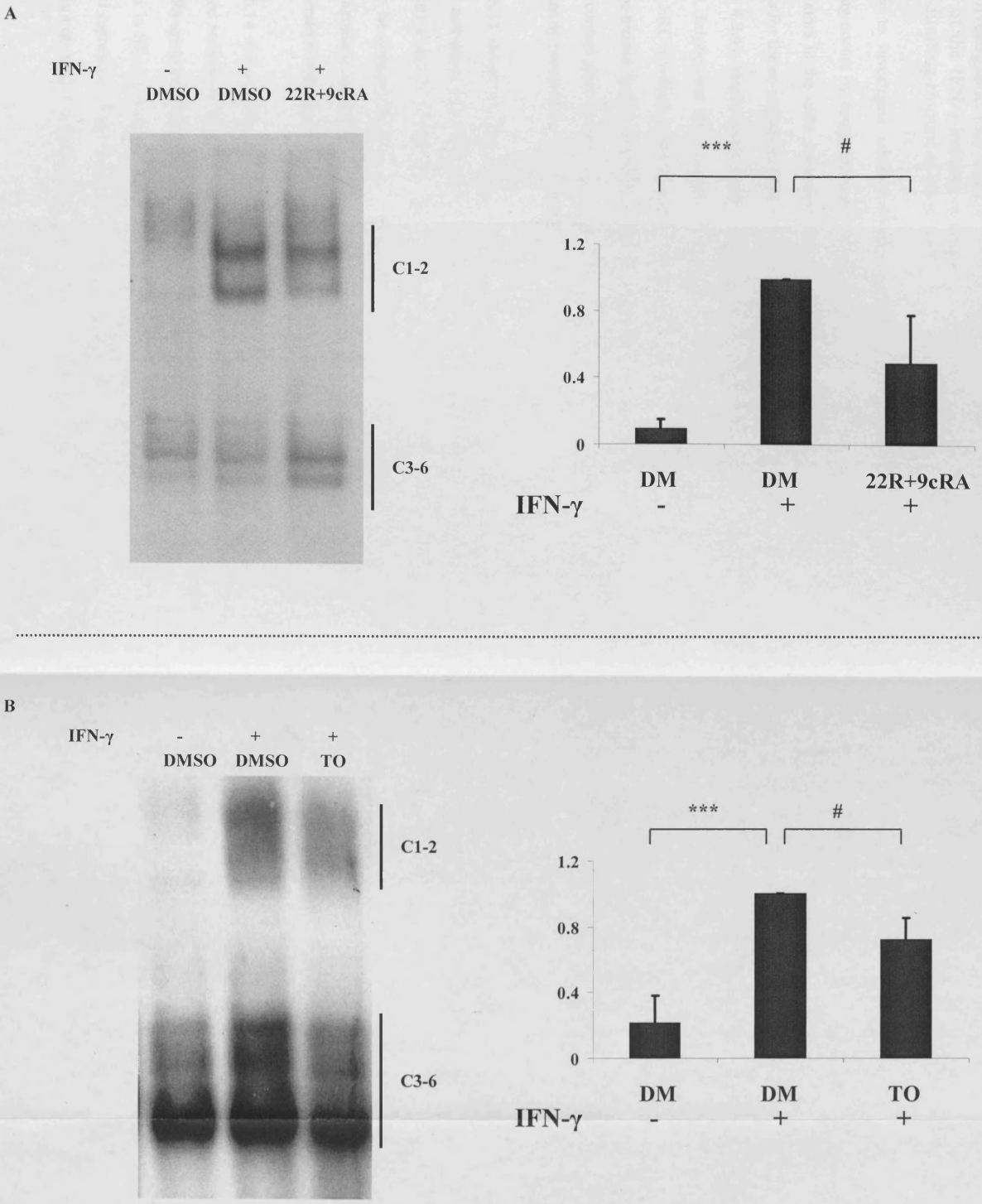


Figure 6.12 Effect of LXR agonists on the IFN-γ-induced STAT1 binding to MCPGAS

PMA-differentiated THP-1 macrophages were pre-treated for 24h with 22R (2μg/ml) and 9cRA (10μM) (A), or for 2h with TO901317 (TO, 10μM) (B), with DMSO (DM) employed as a vehicle-control. The cells were then either stimulated with IFN-γ for 30min or left untreated. Nuclear extracts were prepared as before and used for EMSA using the radiolabelled MCPGAS probe. The images are representative of three independent experiments (left panel). The free probe has been run out of the gel. Densitometric analysis (determined by measuring the intensity of C1-2 in each sample and then calculating the sum value) of these data is presented in the right panel, normalised to the IFN-γ-treated DMSO control. The results shown in the right panels are the mean ratio (± SD) from three independent experiments (***P<0.001, compared to the DMSO-treated control. #P<0.05 compared to the IFN-γ-treated DMSO control).

6.4 Investigation into whether the therapeutic agents mediate repression of IFN-γ signalling by competition for a limiting amount of co-activators in cells

6.4.1 Investigation into whether PPARγ agonists mediate the inhibition of the IFN-γ-induced activation of 3xly6e by competition for a limiting amount of co-activators

In order to investigate whether PPARγ agonists-mediated inhibition of IFN-γ signalling occurs by competition with transcription factors for a limiting amount of co-activators in the cells, co-transfection assays were carried out using expression plasmids for the co-activators SRC-1 and p300. As described in the previous chapter (section 4.3.4), Hep3B cells were co-transfected with the GAS-regulated reporter plasmid, 3xly6e, and DNA constructs specifying for the constitutive expression of p300 or SRC-1 with the empty plasmid vector pcDNA3 used as a control. The cells were pre-treated for 2h with BRL49653 (20μM), 15d-PGJ2 (10μM) or DMSO as vehicle control prior to treatment with IFN-γ (100U/ml) for 6h. Cell extracts were subsequently prepared for luciferase reporter activity assays.

Figure 6.13 shows that the expression of p300 significantly enhanced the IFN-γ-induced activation of 3xly6e, but did not affect the repressive effect mediated by either BRL49653 or 15d-PGJ2. These results suggest that the co-activator p300 is likely to be required for STAT1 transactivation mediated by the cytokine, but the PPARγ agonists-dependent repression of the IFN-γ-mediated STAT1 transactivation is independent of the amount of p300 in the cells.

Figure 6.14 shows that the expression of SRC-1 did not affect either the IFN-γ-stimulated activity of STAT1 response elements or the suppressive effect mediated by PPARγ agonists. These results thus suggest that in contrast to p300, SRC-1 is not involved in STAT1 transactivation in response to IFN-γ and the PPARγ agonists-mediated repression of the IFN-γ-induced STAT1 transactivation is independent of the amount of SRC-1 in the cells.

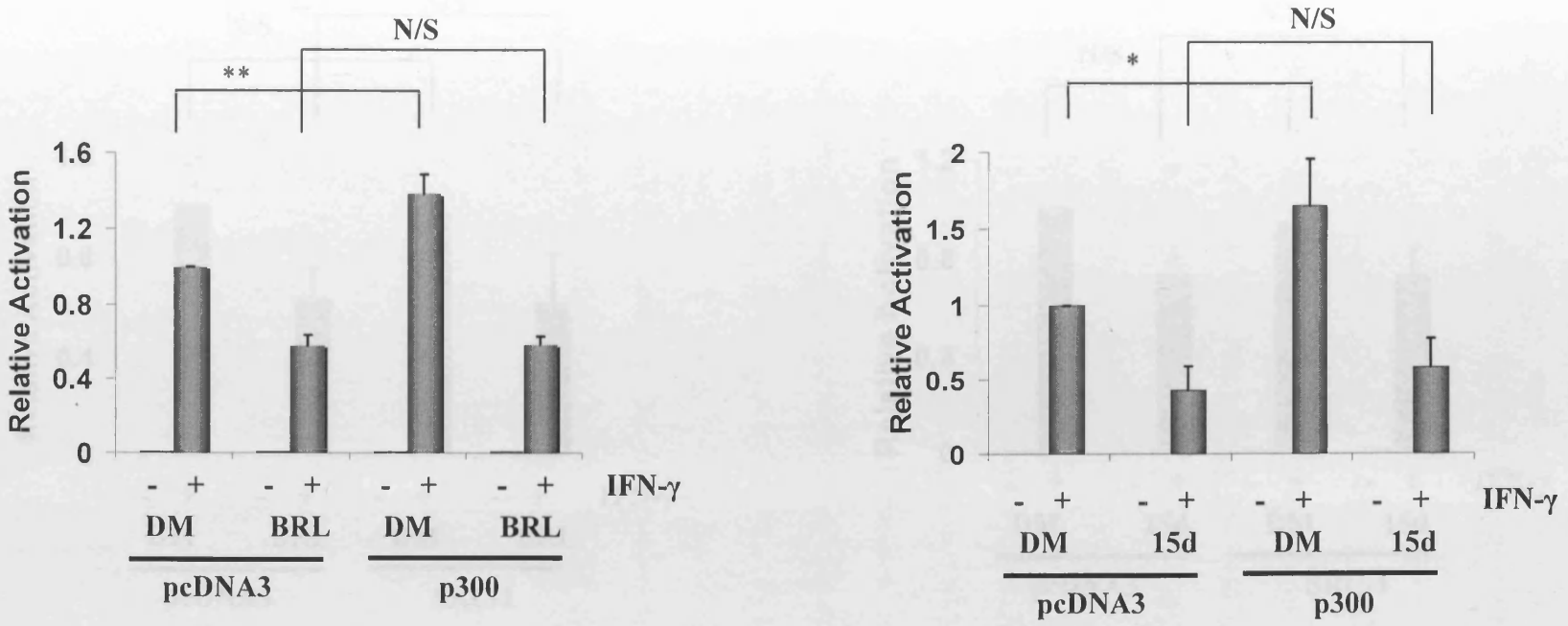


Figure 6.13 Effect of transfection of p300 expression plasmid on the PPARγ agonists-mediated inhibition of IFN-γ-stimulated activity of STAT1 responsive elements

Hep3B cells were co-transfected with 3xly6e and an expression plasmid for p300 or pcDNA3 as the empty vector control. Following 2h incubation with BRL49653 (BRL, 20μM), 15d-PGJ2 (15d, 10μM) or DMSO control (DM), transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assays. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated controls, from three experiments (each carried out in triplicate). The data was further normalised to the relative activation of IFN-γ-treated cells transfected with pcDNA3 (arbitrarily assigned as 1). Student's *t* test was carried out on samples transfected with the p300 plasmid versus IFN-γ-stimulated pcDNA3 transfected samples (**P<0.01; *P<0.05; N/S, not significant)

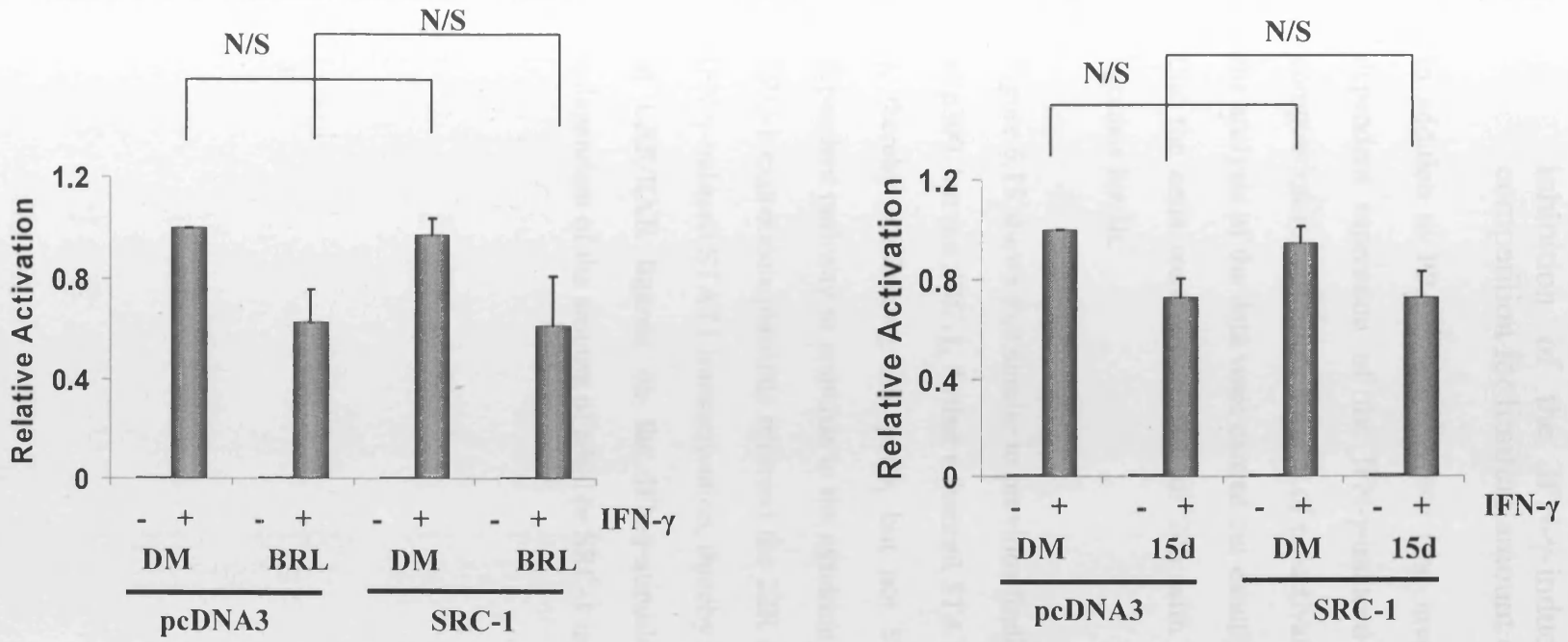


Figure 6.14 Effect of transfection of SRC-1 expression plasmid on the PPARγ agonists-mediated inhibition of IFN-γ-stimulated activity of STAT1 responsive elements

Hep3B cells were co-transfected with 3xly6e and an expression plasmid for SRC-1 or pcDNA3 as empty vector control. Following 2h incubation with BRL49653 (BRL, 20μM), 15d-PGJ2 (15d, 10μM) or DMSO control (DM), transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated controls, from three experiments (each carried out in triplicate). The data was further normalised to the relative activation of IFN-γ-treated cells transfected with pcDNA3 (arbitrarily assigned as 1). Student's *t* test was carried out on samples transfected with the SRC-1 plasmid versus IFN-γ-stimulated pcDNA3 transfected samples (N/S, not significant)

6.4.2 Investigation into whether LXR/RXR agonists mediate the inhibition of the IFN-γ-induced activation of 3xly6e by competition for limiting amount of co-activators

In addition to PPARγ agonists, we next investigated whether the LXR ligands-dependent repression of the IFN-γ-induced STAT1 transactivation occurs by competition for a limiting amount of co-activators in the cells. The experiments and the analysis of the data were carried out exactly as described in section 6.4.1 except that the cells were pre-treated for 24h with 22R with 9cRA instead of PPARγ agonists for 2h.

Figure 6.15 shows that similar to previous findings (Figures 6.13-14), the expression of p300, but not SRC-1, further enhanced STAT1 transactivation in response to IFN-γ, thereby confirming that p300, but not SRC-1, is required for the STAT1-dependent pathway in response to the cytokine. In addition, neither the p300 nor the SRC-1 expression plasmids reversed the 22R and 9cRA-mediated inhibition of the IFN-γ-induced STAT1 transactivation, thereby suggesting that the suppressive effect of LXR/RXR ligands on the IFN-γ-stimulated STAT1-dependent pathway is independent of the amount of p300 or SRC-1 in the cells.

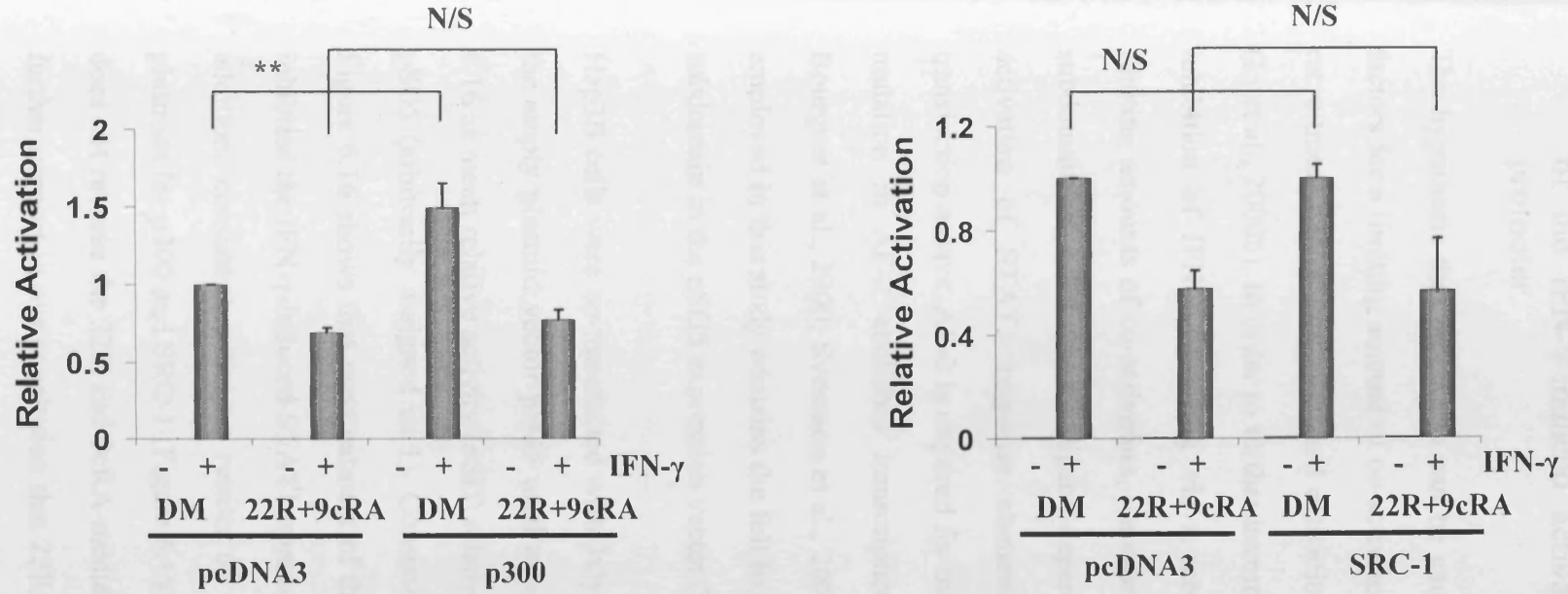


Figure 6.15 Effect of transfection of p300 and SRC-1 expression plasmids on the LXR ligands-mediated inhibition of IFN-γ-stimulated activity of STAT1 responsive elements

Hep3B cells were co-transfected with 3xly6e and expression plasmids for p300 or SRC-1, or pcDNA3 as an empty vector control. Following 24h incubation with 22R (2μg/ml) and 9cRA (10μM) or the DMSO control (DM), transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated controls, from three experiments (each carried out in triplicate). The data was further normalised to the relative activation of IFN-γ-treated cells transfected with pcDNA3 (arbitrarily assigned as 1). Student's *t* test was carried out on samples transfected with the p300 or SRC-1 expression plasmids versus IFN-γ-stimulated pcDNA3 transfected samples (**$P<0.01$; N/S, not significant)

6.4.3 Effect of DN plasmids for LXR on the ligands-mediated inhibition of the IFN-γ-induced activation of 3xly6e and MCP-1 gene promoter

The hypothesis that repression can be caused by competition among transcription factors for a limiting amount of co-activators predicts that deficiency in co-activator recruitment and/or transcriptional activation by LXRs should also affect repression (Li et al., 2000b). In order to further investigate whether 22R and 9cRA mediate the inhibition of IFN-γ signalling via a mechanism dependent on competition for limiting amounts of co-activators, we examined the effects of deletion of the AF-2 subdomain of LXR on the ligand-dependent repression of the IFN-γ-mediated activation of STAT1 response elements and MCP-1 promoter by transient transfection assays. AF-2 is required for the recruitment of co-activators to NRs and mutation in AF-2 abolishes transcriptional activation (Barettino et al., 1994; Bourguet et al., 2000; Svensson et al., 2003). The DN mutant plasmid (DNLXR-α) employed in this study contains the full length sequence of LXR-α lacking the AF-2 subdomain in the pSG5 expression vector (Kocarek et al., 2002).

Hep3B cells were co-transfected with 3xly6e or MCP[213]Luc and DNLXR-α with the empty plasmid vector pSG5 used as a control. Results are presented in Figure 6.16 as mean relative activity (±SD) relative to the induction level in the presence of pSG5 (arbitrarily assigned as 1). Consistent with previous findings (Figure 5.17), Figure 6.16 shows that pre-treatment of the cells with 22R and 9cRA significantly inhibited the IFN-γ-induced STAT1 transactivation and MCP-1 promoter activity. In addition, consistent with our results on co-transfection assays using expression plasmids for p300 and SRC-1 (Figure 6.15), transfection of the cells with DNLXR-α does not reverse the 22R and 9cRA-mediated inhibition. These results thereby lend further support to our conclusion that 22R and 9cRA attenuate IFN-γ signalling via mechanisms independent of competition with transcription factors for a limiting amount of co-activators in the cells.

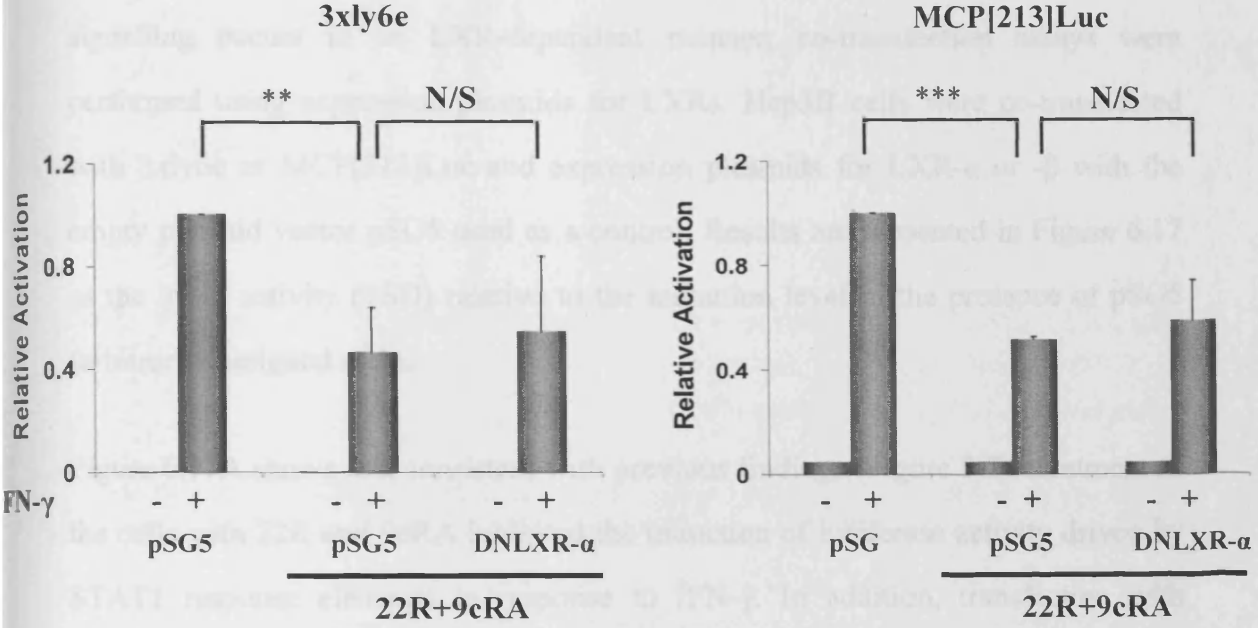


Figure 6.16 Effect of transfection with DNLXR-α on the LXR ligands-mediated inhibition of IFN-γ-stimulated activity of STAT1 responsive elements and MCP-1 promoter

Hep3B cells were co-transfected with 3xly6e or MCP[213]Luc and DNLXR-α. pSG5 was used as an empty vector control. Following 24h incubation with 22R (1μg/ml) and 9cRA (5μM) or DMSO as vehicle control, transfected cells were incubated in the absence or the presence of IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated controls, from three experiments (each carried out in triplicate). The data was further normalised to the relative activation of IFN-γ-treated cells transfected with pSG5 (arbitrarily assigned as 1). Student's *t* test was carried out on samples transfected with DNLXR-α plasmids versus IFN-γ-stimulated pSG5 transfected samples in the presence of the ligands (N/S, not significant). The statistical analysis was also carried out on the ligands treated samples transfected with pSG5 versus IFN-γ-stimulated DMSO control (**P<0.01; ***P<0.001).

6.5 Effect of expression plasmids for LXR on the 22R and 9cRA-mediated inhibition of IFN-γ signalling

In order to investigate whether the 22R and 9cRA-mediated inhibition of IFN-γ signalling occurs in an LXR-dependent manner, co-transfection assays were performed using expression plasmids for LXRs. Hep3B cells were co-transfected with 3xly6e or MCP[213]Luc and expression plasmids for LXR-α or -β with the empty plasmid vector pSG5 used as a control. Results are presented in Figure 6.17 as the mean activity (±SD) relative to the induction level in the presence of pSG5 (arbitrarily assigned as 1).

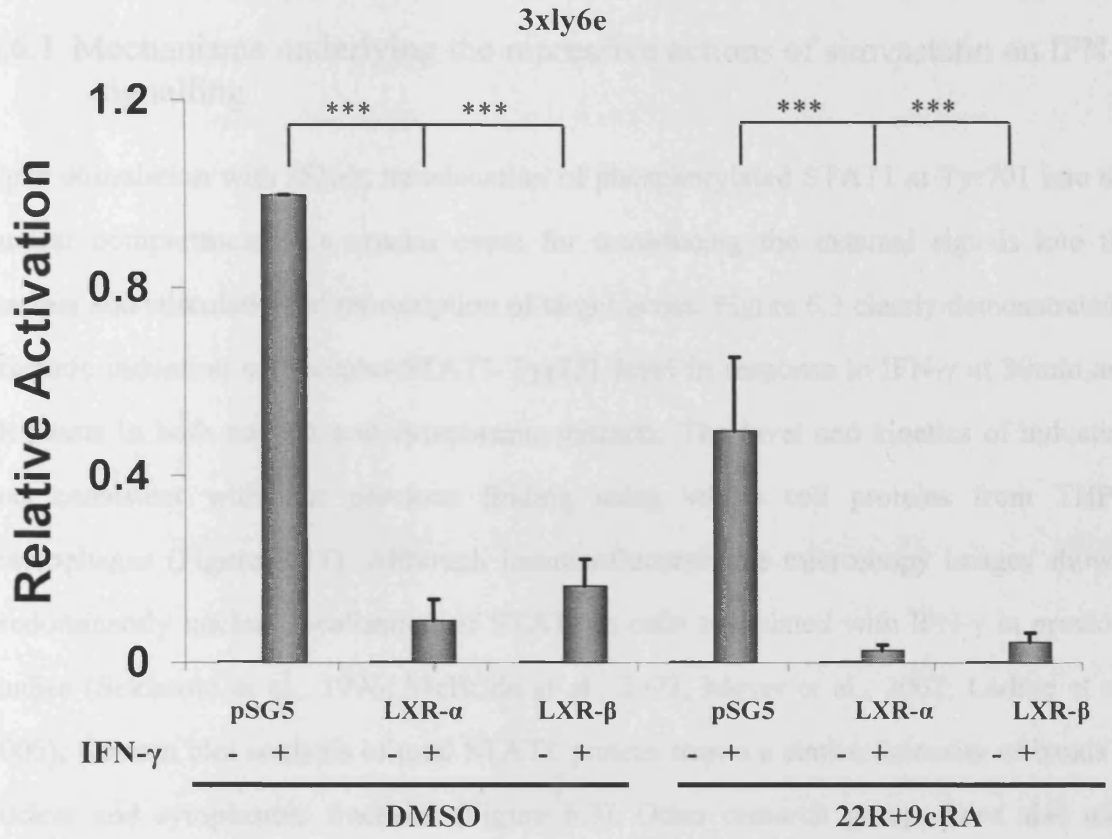
Figure 6.17A shows that consistent with previous findings (Figure 5.7), treatment of the cells with 22R and 9cRA inhibited the induction of luciferase activity driven by STAT1 response elements in response to IFN-γ. In addition, transfection with expression plasmids for LXR-α and -β dramatically suppressed the IFN-γ-induced activity of STAT1 response elements (GAS), in the absence or the presence of 22R and 9cRA. These results thus suggest that the repressive effect on the IFN-γ-stimulated STAT1 transactivation mediated by 22R and 9cRA was dependent on both LXR isoforms, α and β.

In contrast to 3xly6e, Figure 6.17B shows that transfection with expression plasmids of LXR-α and -β did not affect the induction of MCP-1 gene promoter activity in response to IFN-γ with or without treatment of 22R and 9cRA. On the other hand, consistent with the data shown in Figures 5.7 and 6.16, pre-treatment of the cells with 22R and 9cRA inhibited the IFN-γ-induced activation of MCP-1 promoter. These results thereby suggest that the 22R and 9cRA-mediated repressive effect on the IFN-γ-induced activation of MCP-1 gene promoter occurs in a manner independent of LXR.

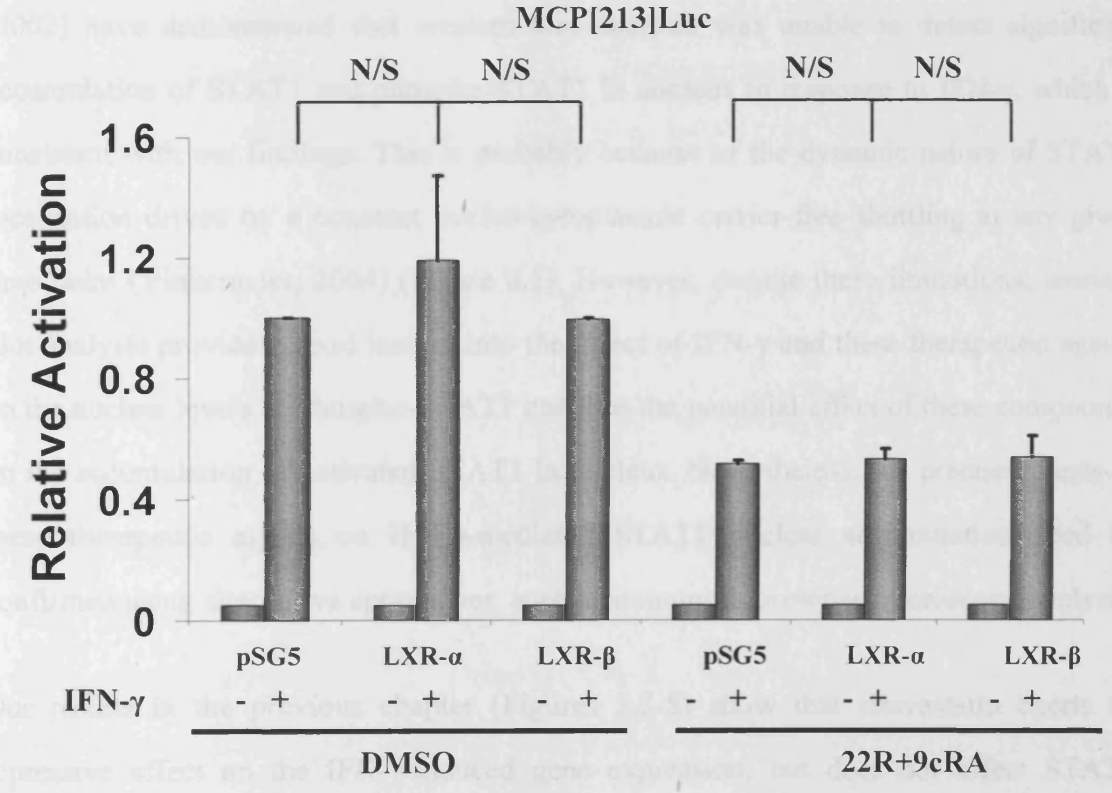
Figure 6.17 Effect of transfection with expression plasmids for LXR-α or -β on the LXR ligands-mediated inhibition of IFN-γ-stimulated STAT1 responsive elements and MCP-1 promoter activity

Hep3B cells were co-transfected with 3xly6e (A) or MCP[213]Luc (B) and expression plasmids for LXR-α or -β. pSG5 was used as an empty vector control. Following 24h incubation with 22R (1μg/ml) and 9cRA (5μM) or DMSO as vehicle control, transfected cells were incubated in the absence or the presence of IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Luciferase counts were normalised to protein concentration and presented as the mean relative activation (±SD) in response to IFN-γ, in relation to unstimulated controls, from three experiments (each carried out in triplicate). The data was further normalised to the relative activation of IFN-γ-treated cells transfected with pSG5 (arbitrarily assigned as 1). Student's *t* test was carried out on samples transfected with over-expression plasmids versus the IFN-γ-stimulated pSG5 transfected samples in the absence or the presence of the ligands respectively (***$P<0.001$; ** $P<0.01$; N/S, not significant).

(A)


3xly6e
Relative Activation
1.2
0.8
0.4
0
***
***
***
***
pSG5
LXR-α
LXR-β
pSG5
LXR-α
LXR-β
IFN-γ
- + - + - + - + - + - +
DMSO
22R+9cRA

(B)


MCP[213]Luc
Relative Activation
1.6
1.2
0.8
0.4
0
N/S
N/S
N/S
N/S
pSG5
LXR-α
LXR-β
pSG5
LXR-α
LXR-β
IFN-γ
- + - + - + - + - + - +
DMSO
22R+9cRA

6.6 Discussion

6.6.1 Mechanisms underlying the repressive actions of simvastatin on IFN-γ signalling

Upon stimulation with IFN-γ, translocation of phosphorylated STAT1 at Tyr701 into the nuclear compartment is a crucial event for transducing the external signals into the nucleus and stimulation of transcription of target genes. Figure 6.3 clearly demonstrated a dramatic induction of phospho-STAT1 Tyr701 level in response to IFN-γ at 30min and 3h points in both nuclear and cytoplasmic extracts. The level and kinetics of induction was consistent with our previous finding using whole cell proteins from THP-1 macrophages (Figure 3.11). Although immunofluorescence microscopy images show a predominantly nuclear localisation of STATs in cells stimulated with IFN-γ in previous studies (Sekimoto et al., 1996; McBride et al., 2002; Meyer et al., 2002; Lodige et al., 2005), western blot analysis of total STAT1 protein shows a similar intensity of bands in nuclear and cytoplasmic fractions (Figure 6.3). Other research groups have also used western blot analysis to investigate the nucleocytoplasmic distribution of STAT1 in response to IFN-γ (Haspel et al., 1996; Meyer et al., 2002). In particular, Mayer et al. (2002) have demonstrated that western blot analysis was unable to detect significant accumulation of STAT1 and phospho-STAT1 in nucleus in response to IFN-γ, which is consistent with our findings. This is probably because of the dynamic nature of STAT1 localisation driven by a constant nucleo-cytoplasmic carrier-free shuttling at any given time point (Vinkemeier, 2004) (Figure 6.1). However, despite these limitations, western blot analysis provide a good insight into the effect of IFN-γ and these therapeutic agents on the nuclear levels of phospho-STAT1 and thus the potential effect of these compounds on the accumulation of activated STAT1 in nucleus. Nevertheless, the precise effects of these therapeutic agents on IFN-γ-mediated STAT1 nuclear accumulation need be confirmed using alternative approaches, such as immunofluoresence microscopy analysis.

Our results in the previous chapter (Figures 5.3-5) show that simvastatin exerts its repressive effect on the IFN-γ-induced gene expression, but does not affect STAT1

phosphorylation in response to this cytokine. EMSA shows that simvastatin does not affect the binding of STAT1 to GAS elements in response to IFN-γ (Figure 6.9). These results together suggest that simvastatin attenuates the IFN-γ-induced gene expression by mechanisms independent of inhibition of STAT1 phosphorylation or DNA binding activity. This conclusion is consistent with a previous study showing that simvastatin attenuates the IFN-γ-induced promoter activation of CIITA gene, but does not affect STAT1 phosphorylation or DNA binding in response to this cytokine in human microvascular endothelial cells (Sadeghi et al., 2001).

Western blot analysis of phospho-STAT1 Tyr701 in nuclear and cytoplasmic fractions shows that simvastatin does not affect the nuclear and cytoplasmic levels of activated STAT1 in the presence of IFN-γ (Figure 6.4). This is consistent with findings that simvastatin does not affect the IFN-γ-stimulated STAT1 phosphorylation in whole cell protein extracts (Figure 5.5B). We therefore suspect that simvastatin does not affect the IFN-γ-stimulated nuclear accumulation of activated STAT1. Consistent with our hypothesis, Sadeghi et al. (2001) have previously shown that simvastatin does not affect the IFN-γ-stimulated nuclear localisation of STAT1 in human microvascular endothelial cells using combination of western blot and immunoflorescence microscopy. Similar approaches could therefore be adopted to confirm such effect of simvastatin in THP-1 macrophages.

6.6.2 Mechanisms underlying the repressive action of PPAR agonists on IFN-γ signalling

Figure 6.10 shows that PPARγ agonists do not have any effect on the binding of STAT1 to GAS elements in response to IFN-γ in THP-1 macrophages. Work presented in the previous chapter (Figures 5.7-10) shows that PPARγ agonists attenuate the IFN-γ-induced gene expression, at least in part, through the inhibition of STAT1 transcriptional activity without targeting STAT1 phosphorylation. These results together suggest that PPARγ agonists repress the IFN-γ-induced STAT1-mediated pathway via mechanism

independent of STAT1 phosphorylation or DNA binding. However, several previous studies show contradictory results. For example, Chen et al. (2003) and Panzer et al. (2008) have demonstrated the inhibitory action of PPARγ agonists on the IFN-γ-dependent STAT1 phosphorylation and DNA binding activities in the mouse macrophage RAW264.7 cell line and the mouse mesangial cells respectively. Such actions have also been confirmed in the rat insulinoma RINm5F cell line (Weber et al., 2003, 2004). Although the exact reasons for the differences between our results and other studies are unclear at present, the cell type- and species-specific actions of PPARγ agonists might have played a role. Indeed, although PPARs have emerged as important regulators of macrophage function in both human and murine species, a number of species-specific differences in terms of mechanism have been reported (Rigamonti et al., 2008). For example, PPARγ activation has been found to decrease the rate of cholesterol esterfication in THP-1 macrophages by reduction of acyl-CoA cholesterol acyltransferase-1 (ACAT-1) mRNA expression (Hirakata et al., 2004). However, in murine macrophages PPARγ has been reported to decrease cholesterol esterfication without affecting ACAT mRNA level (Li et al., 2004). Such species-specificity of PPARs actions indicate that the validation of pharmacological compounds will need to be assessed in humanised model, such as human primary cells, and ultimately in large-scale clinical trails.

Consistent with the results that show that PPARγ agonists do not attenuate the IFN-γ-stimulated STAT1 phosphorylation in whole cell extracts (Figure 5.8), western blot analysis shows that these agonists do not have any effect on the IFN-γ-induced amounts of phospho-STAT1 Tyr701 in the nucleus or the cytoplasm (Figure 6.5). The unchanged level of phospho-STAT1 in the nucleus in isolation suggests that PPARγ agonists are unlikely to affect the nuclear accumulation of activated STAT1 in response to IFN-γ. However, additional experiments using alternative technique such as immunoflourescence microscopy will be required to confirm the hypothesis.

Direct interaction of CBP/p300 and STAT1 has been reported to play essential roles in the JAK-STAT1 pathway induced by IFN-γ and the normal response of macrophages to this cytokine (Zhang et al., 1996; Horvai et al., 1997; Bouhet et al., 2009). Consistent with these previous findings, transfection of the cells with expression plasmids for p300 was found to further enhance the STAT1 transactivation induced by IFN-γ (Figure 6.13). In contrast to p300, transfection with an expression plasmid for SRC-1 did not affect the fold induction of STAT1 transactivation in response to this cytokine (Figure 6.14). Additionally, several previous studies have shown that PPARs repress the activities of transcription factors, such as AP-1 and NFκB by competing for limiting amounts of shared coactivators (Kamei et al., 1996; Li et al., 2000b; Gervois et al., 2001). Figures 6.13-14 show that the expression of p300 and SRC-1 by transfection of the cells with expression plasmids for these co-activators failed to restore the PPARγ agonists-mediated inhibition of IFN-γ-induced STAT1 transactivation. These results thereby suggest that the PPARγ agonists-mediated repression of IFN-γ-induced STAT1 transactivation occurs in a p300- and SRC-1-independent manner. The results are consistent with a previous study that shows that the PPAR-dependent repression of both AP-1- and NFκB-mediated gene expression is independent of the amount of CBP in the cells (Delerive et al., 1999a). The basis for the different results between our study and previous research supporting the co-activator competition model of PPARs (Kamei et al., 1996; Li et al., 2000b; Gervois et al., 2001) is not clear at present. As in most of the cases, conclusions regarding the roles of co-regulators have been based on the use of transient reporter assays involving over-expression of various co-factors that restore repressive effects of PPAR agonists, some researchers have called for studies focusing on the regulation of endogenous genes in a normal chromatin context to further confirm the exact roles of these co-regulators in PPAR agonists-mediated transrepression (Ricote and Glass, 2007).

The molecular mechanisms underlying the repressive actions of PPARα agonists in relation to IFN-γ signalling are poorly understood due to limited research on this aspect. The studies presented in the previous chapter show that PPARα agonist inhibits IFN-γ-

induced gene expression independently of STAT1 phosphorylation. Figure 6.6 shows that the PPARα agonist GW7647 does not affect the nuclear and cytoplasmic level of activated STAT1 in response to IFN-γ. Moreover, the IFN-γ-induced STAT1 binding to GAS elements is also unaffected by the PPARα agonist. These results thereby indicate that the PPARα agonist GW7647 exerts its repressive action on IFN-γ signalling independent of STAT1 phosphorylation or DNA binding activity, and without reducing the nuclear or cytoplasmic amounts of activated STAT1.

As detailed in the General Introduction (section 1.8), Pascual et al. (2005) and Ghisletti et al. (2007) have recently independently shown that in mouse primary macrophages SUMOylation of PPARγ in response to the ligands prevents the clearance of co-repressor complexes by LPS and maintains the repressed state of the inflammatory genes. Interestingly, a parallel SUMOylation-dependent mechanism has also been shown to act in LXR-mediated transrepression (Ghisletti et al., 2007). It will therefore be of great interest to find out whether SUMOylation of PPARs or LXRs is also required for transrepression of IFN-γ signalling.

6.6.3 Mechanisms underlying the repressive actions of LXR agonists in IFN-γ signalling

Western blot analysis of phospho-STAT1 Tyr701 in nuclear and cytoplasmic fraction from THP-1 macrophages shows that the natural LXR and RXR ligands 22R and 9cRA together inhibit the IFN-γ-induced amounts of activated STAT1 in the nucleus, but not in the cytoplasm (Figure 6.7B). The reduced nuclear levels of phospho-STAT1 in isolation suggest that 22R together with 9cRA decreases the IFN-γ-stimulated nuclear amount of activated STAT1. In addition, pre-treatment of the cells with 22R and 9cRA also inhibited the IFN-γ-induced binding of STAT1 to GAS elements on the MCP-1 gene promoter (Figure 6.12A). Nuclear import and retention are known as two steps leading to the nuclear accumulation of activated STAT1 and DNA binding has been shown to be critical for nuclear retention of this transcription factor (section 6.1.1). The reduced DNA

binding activity of STAT1 could therefore result in from its increased dephosphorylation, and subsequently nuclear export. Moreover, results presented in the previous chapter have shown that 22R and 9cRA together inhibited the IFN-γ-induced tyrosine phosphorylation of STAT1 (Figures 5.15-16). As only the tyrosine phsophorylated STAT1 is capable of being actively translocated into the nucleus, inhibition of tyrosine phosphorylation may lead to the reduced cytoplasmic to nuclear translocation of activated STAT1. These results together suggest that the reduced nuclear amount of phospho-STAT1 Tyr701 is likely to be a net result of its inhibited nuclear importation and increased exportation. However, it is worth nothing that the degree of inhibition of the amount of phospho-STAT1 Tyr701 in the nucleus is of similar extent to the decrease observed at the level of whole cell proteins (Figure 5.15). The possibility therefore remains that the reduced nuclear level of phospho-STAT1 is merely because of the inhibition of phosphorylation itself, and that the reduced phosphorylation is simply due to the increased dephosphorylation rate of STAT1 in the nucleus. Alternative approaches such as immunoflorescence microscopy analysis will need to confirm the exact effect of these ligands on the IFN-γ-mediated STAT1 nuclear accumulation.

In contrast to these endogenous ligands, the synthetic LXR agonist TO901317 had no effect on the IFN-γ-induced amount of activated STAT1 in both the nucleus and the cytoplasm (Figure 6.7A). The results are consistent with previous findings using whole cell extracts (Figure 5.14). Interestingly, similar to the combinatorial effect of 22R and 9cRA, TO901317 was shown to inhibit STAT1 binding to GAS elements. These results thereby suggest that synthetic and endogenous agonists of LXR may exert their repressive actions on IFN-γ signalling through distinct mechanisms.

Transfection of the cells with expression plasmids for p300 and SRC-1 failed to overcome the 22R and 9cRA-mediated inhibition of IFN-γ-induced STAT1 transactivation, thereby suggesting that the repressive actions of activated LXR is independent of the amounts of co-activators p300 and SRC-1. The hypothesis that

repression can be caused by competition between different transcription factors for limiting amounts of essential shared coactivators predicts that mutations in LXRs affecting co-activator binding and/or transactivation should also affect transrepression. Therefore, the DNLXR-α plasmid was employed in the investigations. The plasmid contains the full sequence of LXR-α, but lacks the AF-2 subdomain, which is required for the recruitment of co-activators and transcriptional activation (Barettino et al., 1994; Bourguet et al., 2000; Kocarek et al., 2002). Figure 6.16 shows that transfection of the cells with DNLXR-α does not restore the 22R and 9cRA-mediated inhibition of the IFN-γ-induced STAT1 transactivation and MCP-1 promoter activity. These results thereby confirm further that the LXR-mediated transcriptional inhibition occurs via mechanisms different from competition for limiting amounts of shared co-activators in the cells.

It is worth noting that a number of alternative factors might also contribute to the results of the co-transfection assays using DNLXR-α. First, as only DNLXR-α was included in this assay, the inhibitory effect of another isoform, LXR-β in the cells could possibly mask the impact of the dysfunction of LXR-α. Moreover, the expression of LXR-α has been shown to be induced by natural and synthetic LXR agonists in multiple human cell types, including macrophages and the hepatoma Hep3B cell line (Laffitte et al., 2001; Blaschke et al., 2006). 22R and 9cRA-mediated induction of LXR-α expression could possibly overcome the DN effect of the LXR-α mutant. Therefore, more work will be needed to decipher the exact reason(s) why the 22R and 9cRA-mediated inhibition was not affected by transfection of the cells with DNLXR-α.

In the absence or the presence of 22R and 9cRA, transfection of the cells with expression plasmids for LXR-α and -β significantly inhibited the IFN-γ-induced STAT1 transactivation (Figure 6.17A). Additionally, consistent with our previous findings (Figure 5.17), the inhibition in cells transfected with LXR plasmids and treated with these ligands occurred to a greater extent than cells transfected with the LXR plasmids alone. These results thereby suggest that 22R and 9cRA attenuate IFN-γ signalling in LXR-

dependent manner. Interestingly, transfection with LXR-α and -β expression plasmids does not affect the IFN-γ-induced promoter activity of the MCP-1 gene, despite the fact that the activation of the MCP-1 promoter was largely inhibited by the ligands, 22R and 9cRA (Figures 5.17 and 6.17B). These results therefore suggest that the LXR ligands probably exert the repressive effect on MCP-1 expression in an LXR-independent manner. Moreover, the finding that LXR expression plasmids attenuated the IFN-γ-stimulated STAT1 transactivation but not MCP-1 expression is consistent with previous studies that demonstrated a sustained expression of MCP-1 in response to IFN-γ in STAT1-null macrophages (Gil et al., 2001; Ramana et al., 2002). STAT3 has previously been shown to be activated in response to IFN-γ and drive the transcription of certain STAT1 response genes in STAT1-null cells (Qing and Stark, 2004; Ramana et al., 2005). The existence of STAT1 alternative factor(s) may play a role to maintain MCP-1 expression in cells transfected with LXR expression plasmids, which reduce the STAT1 transactivation. However, we cannot rule out the possibility that the complexity of the MCP-1 gene promoter (Figure 3.2) compared to the artificial STAT1 responsive elements might also contribute to the different responses observed on the activation of 3xly6e and MCP[213] plasmids to over-expression of LXR-α or -β in the cells. Further studies will be required to understand the exact molecular basis of the discrepancy between STAT1 transactivation and MCP-1 promoter activation.

As discussed in the previous chapter, the NR ligands could exert their non-genomic actions by activation of intracellular signalling cascades (e.g. MAPK-dependent pathway) through modes either dependent on the classical NRs or novel membrane receptors (section 5.7.4). As discussed above, LXR ligands potentially inhibit the IFN-γ-mediated gene expression, at least in part, in a manner independent of the corresponding receptor. It will be of interest to find out whether the non-genomic effects are responsible for this NR-independent action of LXR ligands. More work will be required to address this issue.

Overall, our results suggest that 22R and 9cRA together attenuate the IFN-γ signalling pathway, at least in part, in a manner dependent on LXR and by inhibition of STAT1 phosphorylation and DNA binding activity, which may together result in decreased nuclear accumulation of activated STAT1. Additionally, the LXR-mediated inhibition occurs via mechanisms different from competition with transcription factors for shared co-activators. LXR-independent mode of action by the ligands might also contribute to the IFN-γ-mediated gene expression. On the other hand, the synthetic LXR agonist TO901317 inhibits the IFN-γ-induced gene expression by attenuating STAT1 DNA binding activity without affecting STAT1 phosphorylation in either the nucleus or the cytoplasm.

CHAPTER SEVEN:

GENERAL DISCUSSION

Chapter 7: General Discussion

7.1 Mechanisms underlying the IFN-γ-mediated regulation of gene expression

Atherosclerosis, an underlying cause of CAD, is an inflammatory disorder orchestrated by cytokines. The pro-inflammatory cytokine IFN-γ is a key regulator of atherogenesis and is involved in all the major steps in the pathogenesis of this disease. The cellular actions and the downstream signalling mechanisms of this important inflammatory mediator have attracted great interest. Extensive studies in last decade have identified a pivotal role for the JAK-STAT pathway in relation to IFN-γ signalling. However, many aspects of IFN-γ-activated JAK-STAT-dependent signalling remain unclear, particularly in relation to the mechanisms involved in the phosphorylation of STAT1 at Ser727, which warrants further studies. The work detailed in this thesis has used several different approaches to delineate the mechanisms underlying the IFN-γ-mediated regulation of gene expression and cellular responses, with particular emphasis on identifying potential targets that may lend themselves to therapeutic intervention of atherosclerosis. The majority of this work has been carried out in macrophages as these cells have a key role in the pathology of atherosclerosis and are an important target for the action of IFN-γ. It was hoped that such studies will not only enhance our understanding of IFN-γ signalling associated with atherosclerosis but, in the long term, may also identify potentially novel therapeutic avenues.

Experiments based on the use of pharmacological inhibitors against potential mediators of IFN-γ signalling identified a key role for ERK and JNK in the phosphorylation of STAT1 Ser727 in response to this cytokine. The crucial role of these kinases in IFN-γ-activated signalling was subsequently substantiated through the use of kinase assays, and transfection experiments using DN mutants. The work also

identified important roles for other kinases (CK2, PI3K, PKC-δ and CaMKII) in the IFN-γ-mediated regulation of expression of a range of model genes in macrophages. These kinases were found to mediate the regulation of gene expression in response to IFN-γ, at least in part, through modulation of STAT1 Ser727 phosphorylation.

7.1.1 Roles of ERK and JNK in the IFN-γ-mediated signalling pathway in macrophages

The ERK inhibitors (PD98059 and U0126) and the JNK inhibitor (SP600125) markedly inhibited the phosphorylation of STAT1 at Ser727 in response to IFN-γ in THP-1 macrophages. These results strongly suggest key roles for ERK and JNK in the IFN-γ-stimulated activation of STAT1 via regulation of phosphorylation at Ser727. The important roles of these two kinases in mediating the IFN-γ-stimulated STAT1 phosphorylation were also extended to human primary monocyte-derived macrophages and human hepatocytes through the use of pharmacological inhibitors. These results thereby suggest that the use of ERK and JNK as key regulators of STAT1-dependent signalling occurs in both human macrophages and hepatocytes.

Transfection based assays, in Hep3B cells, confirmed that ERK and JNK, as well as other key components (Ras, Raf-1 and MEK1) of these pathways were also involved in the IFN-γ-induced STAT1 transactivation. The inhibition also occurred at the level of IFN-γ-induced activation of MCP-1 promoter as determined by the use of DN mutants in transfection assays. RT-PCR experiments further confirmed a crucial role of ERK in the regulation of gene expression downstream of STAT1 in response to this cytokine. Thus, the ERK inhibitor, PD98059 inhibited the IFN-γ-induced expression of MCP-1, MIP-1β, IP-10, SOCS-1 and ICAM-1, all of which have previously been shown to be primarily regulated through a STAT1-dependent pathway by this cytokine. These results together suggest that ERK and JNK-dependent pathways regulate the IFN-γ-mediated gene expression associated with atherosclerosis, at least in part through the modulation of STAT1 phosphorylation in human macrophages,

particularly at Ser727. Consistent with our findings, Overbergh et al. (2006) have shown that in PMA-differentiated THP-1 macrophages, ERK is responsible for the IFN-γ-induced expression of 1,25-dihydroxyvitamin D3, through regulation of STAT1 Ser727 phosphorylation. In addition, JNK has been shown to regulate the expression of MCP-1 in response to IFN-γ and LPS by modulation of STAT1 Ser727 phosphorylation in murine dermal fibroblast (Yamana et al., 2009). However, whether ERK and JNK activate STAT1 through downstream effector kinases or by a direct action remains to be determined.

In addition to STAT1-dependent mechanisms, alternative pathways have also been previously reported to regulate the expression of MCP-1 and MIP-1β genes in response to IFN-γ (Gil et al., 2001; Ramana et al., 2001). We can therefore not rule out the possibility that ERK and JNK are also involved in STAT1-independent pathways along with its action on STAT1 activity. Indeed, Gough et al. (2007) have recently demonstrated that ERK1/2 is activated independently of the JAK1-STAT1 signalling pathway and is responsible for the IFN-γ-induced expression of iNOS gene in murine embryonic fibroblasts. The possibility therefore remains that these two kinases utilise different mechanism for each of these genes.

Results from kinase assays add further support to the important roles of these two kinases in IFN-γ-dependent signalling. Treatment of the cells with IFN-γ resulted in a 3- and 1.5-fold induction in the activity of ERK and JNK respectively, which was consistent with the kinetics of STAT1 phosphorylation and induction of gene expression in response to this cytokine.

It is worth noting that recent studies have shown that pharmacological inhibitors for ERK1/2 (e.g. PD98059 and U0126) can also attenuate the activation of MEK5 and subsequently ERK5, although higher concentrations are required (Mody et al., 2001). In HeLa cells, the concentrations of these inhibitors required for inhibiting the ERK5

pathway (100μM for PD90859 and 10μM for U0126) is 10-fold higher than that required for inhibiting ERK1/2 (10μM for PD98059 and 1μM for U0126) (Mody et al., 2001). We have shown in human macrophages and hepatocytes that PD98059 (at 5, 30 and 50μM) and U0126 (at 5, 10 and 20μM) inhibited the IFN-γ-induced STAT1 phosphorylation at all these concentrations. Inhibition of the IFN-γ-induced gene expression by PD98059 at these concentrations has also been demonstrated.

7.1.2 Roles of other kinases in IFN-γ-mediated signalling pathways in macrophages

In addition to ERK and JNK, work presented in this thesis has identified important roles for other kinases in the IFN-γ-mediated signalling pathway in relation to STAT1 phosphorylation. These kinases include CK2, PI3K, PKC-δ and CaMKII.

Previous studies in our laboratory have identified critical roles for CK2 and PI3K in the IFN-γ-regulated expression of several genes in mouse macrophages (Harvey et al., 2007). Work detailed in this study have extended the investigation into human macrophages and confirmed important roles of these two kinases in IFN-γ signalling in THP-1 macrophages. Consistent with previous findings in mouse macrophages, pre-treatment of THP-1 macrophages with inhibitors for CK2 (apigenin) and PI3K (LY294002) attenuated the IFN-γ-induced expression of MCP-1 and STAT1 phosphorylation at Ser727, without affecting that at Tyr701. Previous findings by our research group based on RT-PCR analysis of a number of marker genes, along with gene expression profiling, have shown that CK2 and PI3K are involved in the regulation of a range of genes important for inflammation and atherosclerosis in response to IFN-γ in mouse macrophages (Harvey et al., 2007). These results together suggest an important role for CK2 and PI3K in the regulation of IFN-γ responsive gene expression in macrophages, at least in part, via modulation of STAT1 phosphorylation at Ser727.

Inhibitors for PKC-δ (rottlerin) and CaMKII (KN93) have both been shown to attenuate the IFN-γ-induced STAT1 phosphorylation at Tyr701 and Ser727, thereby suggesting that these two kinases mediate the IFN-γ-induced STAT1 activity via regulation of phosphorylation at Tyr701 and Ser727. The key roles of PKC-δ in the IFN-γ-mediated regulation of gene expression was also demonstrated by RT-PCR analysis that showed that rottlerin inhibited the expression of MCP-1, ICAM-1, SOCS-1 and ICERI/II in the presence of this cytokine. Similar roles of these two kinases in IFN-γ signalling have been previously demonstrated by other research groups. PKC-δ has been shown to regulate the expression of some IFN-γ-mediated gene (e.g. CIITA) through modulation of STAT1 Ser727 phosphorylation in human leukaemia cell line (Deb et al., 2003) and mouse macrophage cell line RAW264.7 (Kwon et al., 2007). CaMKII has also been shown to be required for the IFN-γ-mediated STAT1 Ser727 phosphorylation in mouse fibroblasts (Nair et al., 2002).

7.2 Mechanisms underlying the inhibitory effects of therapeutic agents on IFN-γ signalling

Ligands for NRs, PPARs and LXRs, as well as cholesterol-lowering drugs, statins have recently been found to exert profound anti-inflammatory and protective effects in the development of atherosclerosis. Despite their well established significance in inflammatory responses and potential clinical benefits in atherosclerosis, the molecular mechanisms underlying such anti-inflammatory and athero-protective effects remain poorly understood, particularly in relation to IFN-γ actions and the JAK-STAT pathway. Work presented in this thesis has used several different approaches to decipher the molecular basis of repression of IFN-γ-induced gene expression by these therapeutic agents, with particular focus on their actions on the JAK-STAT pathway. The majority of this work was carried out in macrophages as these cells are an important target for the anti-inflammatory actions of PPARs and LXRs, as well as the pleiotropic effects of statins. It was hoped that such studies will not only enhance our understanding of the molecular mechanisms underlying the anti-inflammatory and atheroprotective effects of these agents, but may also benefit the development of effective and safe therapeutic intervention of this disease in the future. The results of these studies are summarised in Table 7.1 and detailed in following sections.

7.2.1 Mechanisms underlying the PPARs-mediated inhibitory effects on IFN-γ signalling

Pre-treatment of the cells with the PPARγ agonist (BRL49653) attenuated the IFN-γ-induced expression of MCP-1, IP-10, MIP-1β, CIITA pIII and SOCS-1. Similarly, the PPARα agonist (GW7647) prevented the expression of these genes along with CIITA pIV and ICERI/II. The inhibitory effect of PPARγ agonists (BRL49653 and 15d-PGJ2) on the IFN-γ-stimulated transcription of the MCP-1 gene was also confirmed by transient transfection assays. The study was subsequently extended to delineate the potential mechanisms.

Table 7.1 Summary of results from investigation of the mechanisms underlying the inhibitory effects of therapeutic agents on the IFN-γ-activated, STAT1-dependent signalling pathway

	NR targeted	MCP-1 promoter activation	STAT1 trans -activation	STAT1 phosphorylation	Nuclear amount of activated STAT1	STAT1 DNA binding activity
Simvastatin		↓	↓	→	→	→
BRL49653	PPARγ	↓	↓	→	→	→
15d-PGJ2	PPARγ	↓	↓	→	→	→
GW7647	PPARα	/	/	→	→	→
TO901317	LXR	/	/	→	→	↓
22R+9cRA	LXR	↓	↓	↓	↓	↓

→, no effect; ↓, inhibition; /, not investigated

Abbreviations: STAT, signal transducers and activators of transcription; MCP, monocyte chemoattractant protein; 22R, 22(R)-hydroxycholesterol; 9cRA, 9-*cis* retinoic acid; PPAR, peroxisome proliferators-activated receptor; LXR, liver X receptor; NR, nuclear receptor

Transient transfection assays showed that PPARγ agonists (BRL49653 and 15d-PGJ2) attenuated the IFN-γ-induced STAT1 transactivation. Western blot analysis showed that none of the PPARs agonists employed in this study (BRL49653, 15d-PGJ2 and GW7647) affected the IFN-γ-stimulated STAT1 phosphorylation or nuclear amount of activated STAT1. EMSA analysis additionally showed that these compounds failed to affect the STAT1 DNA binding activity. The results therefore suggest that the PPARγ agonists prevent the IFN-γ-induced gene expression, at least in part, by inhibiting STAT1-dependent pathway, without affecting STAT1 phosphorylation, DNA binding activity or nuclear amount of activated STAT1. Transfection of the cells with expression plasmids for p300 or SRC-1 failed to restore the PPARγ agonists-mediated inhibition of IFN-γ-stimulated STAT1 transactivation, thereby suggesting that PPARγ agonists-mediated transrepression of STAT1 transcriptional activation potential is independent of the amount of p300 or SRC-1 in the cells.

7.2.2 Mechanisms underlying the LXRs-mediated inhibitory effects on IFN-γ signalling

RT-PCR analysis showed that treatment of the cells with the LXR agonist TO901317 resulted in a slight but reproducible inhibition of the IFN-γ-induced expression of MCP-1 and CIITA pIV. The LXR agonist-dependent repression of MCP-1 gene transcription was also confirmed by transfection assay using endogenous LXR/RXR ligands 22R and 9cRA. In addition, these ligands together attenuated the IFN-γ-stimulated STAT1 transactivation. These results together suggest the LXR ligands attenuate the expression of IFN-γ response genes, at least in part, via their inhibitory actions on the STAT1 transcriptional activation potential. Further studies focused at delineating the potential mechanisms involved in the inhibitory actions of LXR ligands on STAT1-dependent signalling.

Western blot analysis and EMSA showed that 22R and 9cRA attenuated the IFN-γ-stimulated STAT1 phosphorylation and binding to GAS element. Moreover, western blot analysis on nuclear and cytoplasmic fractions showed that these ligands reduced the amount of phospho-STAT1 Tyr701 in the nucleus, but not in the cytoplasm. It has been widely accepted that IFN-γ stimulates the nuclear accumulation of activated STAT1 (section 6.1.1) and such accumulation can be visualised using immunoflurescence microscopy (Higashi et al., 2003). The reduced amount of phospho-STAT1 in the nucleus thereby suggests a potential inhibitory effect of 22R and 9cRA on nuclear accumulation of activated STAT1. Such inhibition is probably as a result of inhibited STAT1 phosphorylation and DNA binding activity, which decrease the nuclear import of activated STAT1 and increase the nuclear export of dephosphorylated STAT1 respectively. However, we, at present, do not rule out the possibility that the reduced amount of phospho-STAT1 in the nucleus is merely because of the inhibition of its phosphorylation, and vice versa, that the reduced phosphorylation is simply due to the increased dephosphorylation rate of STAT1 in nucleus. More work will therefore be required to confirm the exact role of LXR

ligands on STAT1 nucleo-cytoplasmic translocation.

Over-expression of co-activators p300 and SRC-1 did not restore the inhibition of the IFN-γ-induced STAT1 transactivation by 22R and 9cRA, thereby suggesting that the LXR ligands mediate the inhibition of STAT1 transcriptional activation potential through mechanisms independent of competition for co-activators p300 and SRC-1 in the cells. In addition, transfection of the cells with DNLXRα does not overcome the 22R and 9cRA-mediated inhibition of IFN-γ-induced STAT1 transactivation and MCP-1 promoter activation. The DNLXRα is known to be deficient in recruitment of co-factors. Work previously carried out in our laboratory has confirmed that transfection of the cells with DNLXRα attenuates the LXR ligands-mediated activation of the ABCA1 promoter (Huwait, 2008). These results therefore add further support to the notion that the 22R and 9cRA-mediated transrepression of IFN-γ signalling occurs through mechanisms distinct from competition for a limiting amount of co-activators in the cells.

Transient transfection assays using expression plasmids specifying for LXR-α and -β showed that 22R and 9cRA attenuated the IFN-γ-stimulated, STAT1-dependent signalling in a manner dependent on LXR isoforms. In the absence of LXR ligands, transfection of the cells with expression plasmids specifying for LXR-α and -β dramatically inhibited the IFN-γ-stimulated STAT1 transactivation. In addition, the inhibition was more extensive in the presence of LXR ligands. In contrast, transfection with these expression plasmids did not affect the IFN-γ-induced activation of MCP-1 promoter in the presence or the absence of 22R and 9cRA, thereby suggesting that the LXR ligands potentially inhibit MCP-1 gene expression through an LXR-independent mechanism.

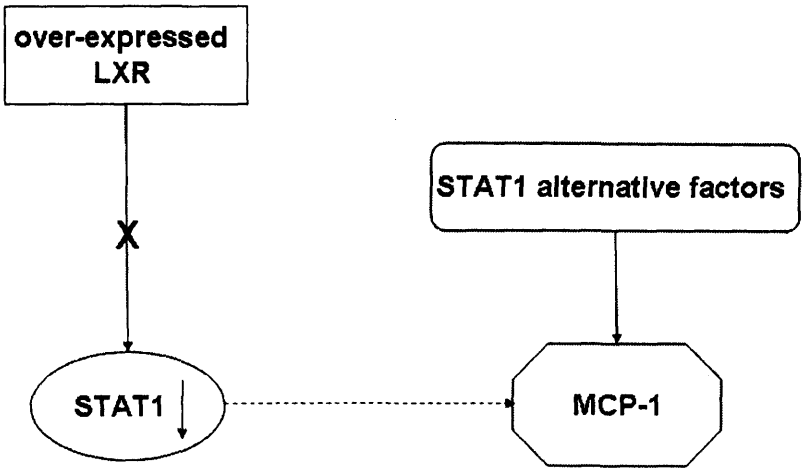
The exact molecular basis for the discrepancy observed between STAT1 transactivation and MCP-1 promoter activation remains to be determined. However,

taking together all the findings on LXR ligands-mediated inhibition of IFN-γ responses and the potential underlying mechanisms identified during the course of this study, we propose a model (Figure 7.1) here that provides a potential explanation. According to this model, in the absence of the LXR ligand, over-expressed LXRs (as a result of transfection with expression plasmids in this case) attenuate the IFN-γ-stimulated STAT1 transactivation. Previous studies have suggested the potential roles of factors independent of STAT1 in the IFN-γ-mediated regulation of MCP-1 (Gil et al., 2001; Ramana et al., 2002). More recently, STAT3 has been found to be activated and replace the STAT1 in the STAT1-deficient cells to mediate the transcription of certain genes in response to IFN-γ (Qing and Stark, 2004; Ramana et al., 2005). It is thus tempting to speculate that as a result of inhibition of STAT1 transcriptional activity by LXRs, other factors become activated and mediates the transcription of the MCP-1 in response to IFN-γ (Figure 6.17B). This might explain why the activation of MCP-1 promoter remained unchanged following transfection with the LXR expression plasmids in the absence of the ligands, despite the marked inhibition of STAT1 transactivaiton (Figure 6.17). Following stimulation with the ligands, activated LXRs inhibit STAT1 phosphorylation and transactivation. In addition to its LXR-dependent mode of action, the LXR ligands could potentially modulate other factors, and thereby MCP-1 gene transcription, in a non-genomic manner that is potentially independent of LXRs. As a result, these LXR-dependent and -independent actions of ligands lead to a reduction of the IFN-γ-induced MCP-1 expression (Figure 7.1C). This model is consistent with our findings that MCP-1 promoter activation is inhibited by 22R and 9cRA, and that MCP-1 expression is decreased by TO901317. Overall, according to this model, the LXR agonists could exert their transrepression on IFN-γ responsive gene, via both LXR-dependent and -independent mechanisms. In contrast, the inhibitory action of LXR agonists on STAT1 transactivation is primarily dependent on LXR isoforms. Such dual actions of the ligands could potentially explain the discrepancies between STAT1 transactivation

and MCP-1 expression, as well as the different responses of MCP-1 expression to LXR ligands and over-expression of LXRs in the cells.

Non-genomic actions are increasingly being recognised as important contributors to the effects of ligands of NR in the regulation of cellular responses. One of the important features of non-genomic action is that it occurs rapidly, usually in the seconds to minutes range (Losel and Wehling, 2003). As observed in this study, the inhibitory effect of 22R and 9cRA on STAT1 phosphorylation, nuclear level, DNA binding activity and transactivation occurs at the 30min point following treatment of THP-1 macrophages with these ligands. Such a rapid response implies the potential involvement of non-genomic actions in the modulation of STAT-dependent pathway by LXR ligands. More work will be needed to confirm this hypothesis. Moreover, in addition to classical NRs, novel non-classical, membrane-associated receptors are emerging as key players for the NR ligands in mediating the non-genomic actions in some cases (Wehling and Losel, 2006). It will be thus of great interest to delineate whether a non-genomic action is involved in such transrepression.

(B) In the cells with action of LXRs, but without LXR ligands



(C) In the cells stimulated with LXR ligands

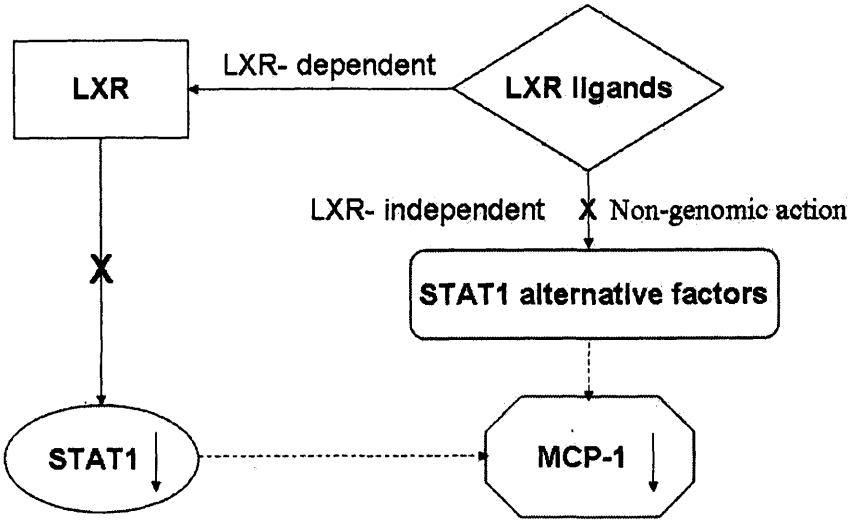


Figure 7.1 A putative model of LXR-dependent and -independent actions of ligands on IFN-γ-a and gene expression

IFN-γ activates STAT1, which in turn mediates the expression of MCP-1 (A). The over-e IFN-γ-stimulated STAT1 transactivaiton. As a result, STAT1 alternative factors become activated a the transcription of MCP-1 gene. Expression of MCP-1 thus remains unchanged (B). LXR ligands p IFN-γ responsive gene expression through LXR-dependent and -independent mechanisms (the non-genomic action). On one hand, the ligands activate the LXRs, which in turn repress the ST potential. On the other hand, the ligands could also target the alternative factors independently of LXR MCP-1 is thus reduced as a result of both STAT1-dependent and independent pathways being inhibite ↓, activation or expression inhibited; →, expression remained; solid arrow, positive regulation; solid a dot arrow, pathway being inhibited

Abbreviations: LXR, liver X receptor; MCP, monocyte chemoattractant protein; STAT, signal transducer and a

7.2.3 Mechanisms underlying the simvastatin-mediated inhibitory effects on IFN-γ signalling

RT-PCR analysis has clearly shown that simvastatin inhibits the IFN-γ-induced expression of MCP-1, IP-10, MIP-1β, ICAM-1, SOCS-1 and CIITA pIV, but not CIITA pIII, thereby suggesting a selective action on IFN-γ responsive gene expression. The inhibitory effect of simvastatin on MCP-1 transcription was further confirmed using transient trasfection assays that showed that the IFN-γ-induced MCP-1 promoter activation was reduced by simvastatin. Transfection assays additionally demonstrated a decrease in STAT1 transactivation in the presence of IFN-γ following treatment of the cells with simvastatin. These results together suggest that simvastatin mediates the inhibition of the IFN-γ responsive gene expression, at least in part, by attenuating STAT-1-dependent pathway. Further studies were therefore focused on the mechanisms underlying such an inhibitory effect of simvastatin.

Western blot analysis using whole cell extracts along with nuclear and cytoplasmic proteins showed that simvastatin does not affect the IFN-γ-stimulated STAT1 phosphorylation or nuclear amount of Tyr701 phospho-STAT1. EMSA also indicated that simvastatin had no effect on the IFN-γ-induced STAT1 DNA binding activity. These results together suggest that simvastatin exerts the inhibitory effect on IFN-γ-induced gene expression by attenuating STAT-1 transactivation without affecting the phosphorylation, nuclear accumulation or DNA binding activity of this transcription factor. More work will therefore be required to elucidate the exact mechanisms by which simvastatin inhibits IFN-γ signalling.

Previous studies have suggested that statins attenuate the expression of many inflammatory response genes, such as iNOS and MCP-1, via PPARs (Paumelle et al., 2006; Yano et al., 2007). Interestingly, we noticed that simvastatin and the PPARγ agonist (BRL49653) both inhibited the IFN-γ-induced expression of MCP-1 and SOCS-1, while simvastatin and the PPARα agonist (GW7647) both inhibited the

expression of MCP-1, SOCS-1 and CIITA pIV. It is thus tempting to speculate that the inhibitory effects of simvastatin on the expression of these genes are probably mediated via PPARs. The cross-talk between statins and PPARs may thus represent a potential avenue for future work as a result of this study.

A recent study by Montecucco et al. (2009) has shown that simvastatin inhibits C-reactive-protein (CRP)-induced chemokine secretion and expression of ICAM-1 in human primary monocytes, as well as the chemotactic migration of these cells through the attenuation of the ERK1/2 pathway. Activation of the ERK1/2 pathway has been shown to be responsible for the expression of a range of inflammatory response genes (i.e. MCP-1, IP-10, MIP-1β and ICAM-1) in response to IFN-γ in this study (Chapter 4). It would thus be of great interest to identify whether a similar ERK-dependent mechanism is also involved in the inhibitory effect of simvastatin on IFN-γ-induced gene expression.

In summary, the work presented in this thesis has been successful in enhancing our understanding of the signalling cascades by which IFN-γ regulates gene expression, in relation to STAT1-dependent pathway, as well as the molecular basis underlying the statin- and agonists for NRs (PPARs and LXRs)-mediated inhibition of IFN-γ responsive gene expression in THP-1 macrophages. The major key findings are as follows:

1. Kinases, including ERK, JNK, CK2, PI3K, PKCδ and CaMKII play important roles in the stimulation of STAT1 Ser727 phosphorylation in response to IFN-γ;

2. ERK and JNK-dependent pathways play key roles in the IFN-γ-stimulated STAT1 transactivation and MCP-1 promoter activity;

3. ERK acts as an important mediator in the regulation of gene expression (i.e. MCP-1, MIP-1β, IP-10, ICAM-1 and SOCS-1) in response to IFN-γ;

4. ERK and JNK activity is induced by IFN-γ by about 3- and 1.5-fold respectively;

5. PPARα agonist inhibits IFN-γ-induced gene expression (e.g. MCP-1) without affecting STAT1 phosphorylation, DNA binding activity or nuclear amount of activated STAT1;

6. PPARγ agonists inhibit the IFN-γ-induced gene expression (e.g. MCP-1), at least in part, by attenuating STAT1-dependent pathway without affecting STAT1 phosphorylation, DNA binding activity or nuclear amount of activated STAT1;

7. LXR agonists inhibit the IFN-γ-induced gene expression (e.g. MCP-1), at least in part, by attenuating STAT1-dependent pathway through reduction of

STAT1 phosphorylation, DNA binding activity and potentially nuclear accumulation;

8. Inhibition of IFN-γ responsive gene transcription by agonists for PPARγ and LXRs does not occur via competition for a limiting amount of shared co-activators, SRC-1 or p300 with transcription factors in the cells;

9. LXR agonists-mediated inhibition of the IFN-γ-stimulated STAT1 transactivation occurs in a mode dependent of NR LXRs, while the inhibition of MCP-1 gene transcription occurs, at least in part, independently of LXRs;

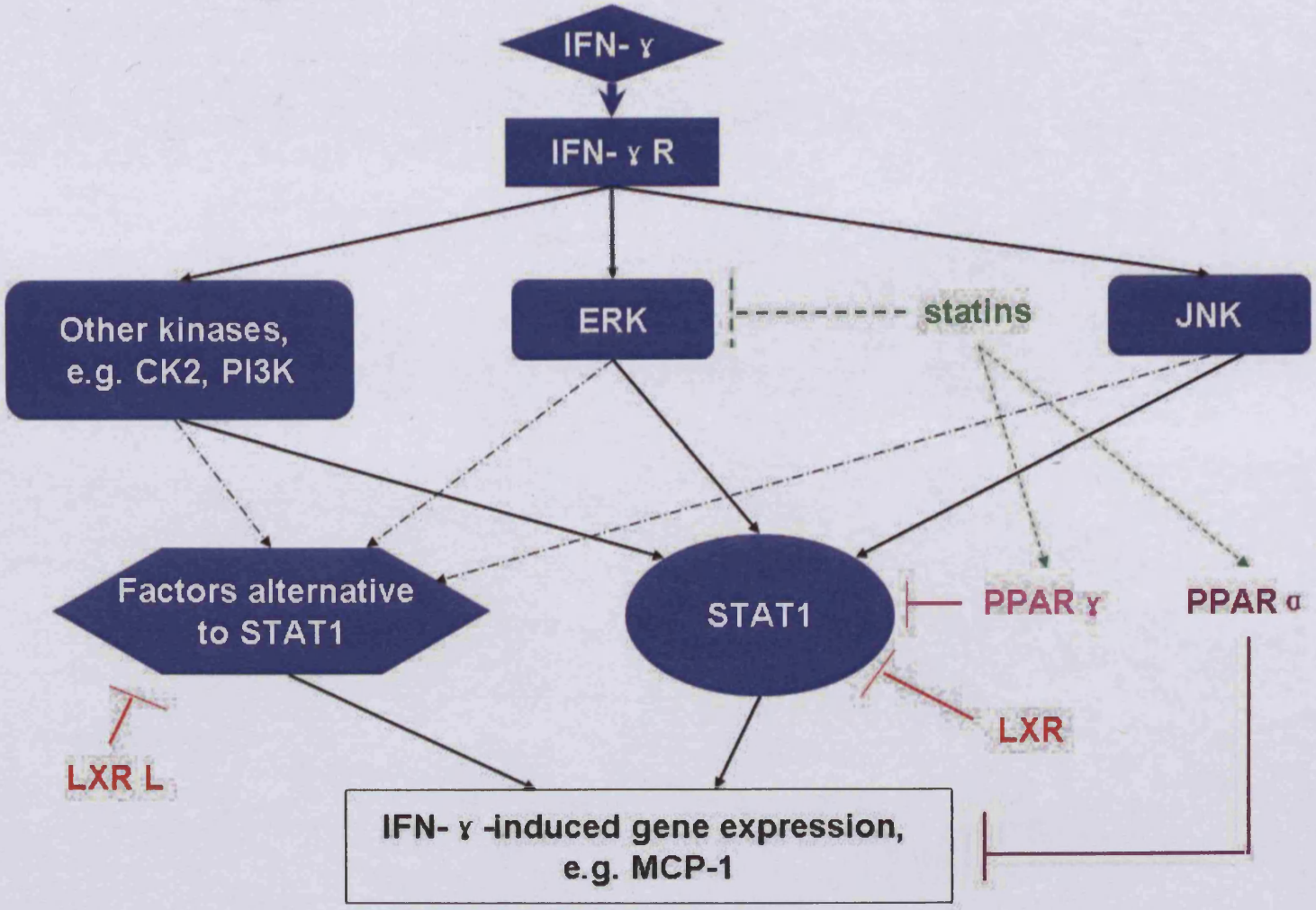
10. Simvastatin inhibits the IFN-γ-induced gene expression (i.e. MCP-1), at least in part, by attenuating the STAT1-dependent pathway without affecting STAT1 phosphorylation, DNA binding activity or nuclear amount of activated STAT1.

The different pathways for the IFN-γ-mediated regulation of gene expression in human macrophages and the actions of agonists for PPARs and LXRs, as well as statins on these pathways are illustrated in Figure 7.2.

Figure 7.2 Different pathways in the IFN-γ-mediated regulation of gene expression and the putative model for the actions of statins, PPARs and LXRs on these pathways, particularly in relation to STAT1 transcriptional activity

A number of kinases, including ERK, JNK, CK2, PI3K, PKCδ and CaMKII play crucial roles in the IFN-γ-mediated regulation of gene expression, at least in part, through modulation of STAT1 transcriptional activity (black solid arrows). The putative roles for these kinases in STAT1-independent pathways are denoted as black dot arrows and require further study. Agonists for PPARs and LXRs inhibit the IFN-γ-induced gene expression (e.g. MCP-1). Ligand-dependent PPARγ (pink) and LXR (red) exert the inhibitory effects by attenuating STAT1 transcriptional activity. Additionally, LXR ligands may also target alternative factor(s) to STAT1 in an LXR-independent manner. Statins (green) inhibit the IFN-γ-induced gene expression (e.g. MCP-1) through novel mechanisms, probably via potentiating the transrepression of PPAR-γ and -α, or attenuating the ERK pathway. Such putative actions of statins (represented as green dot arrows) are derived from previous publications (see text) and require further evaluation in human macrophages.
→, positive regulation; —|, antagonism; ---, putative action

Abbreviations: IFN, interferon; IFN-γR, interferon-gamma receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor; LXR L, LXR ligand; MCP, monocyte chemoattractant protein; STAT, signal transducer and activator of transcription


IFN- γ
IFN- γ R
Other kinases,
e.g. CK2, PI3K
ERK
statins
JNK
Factors alternative
to STAT1
STAT1
PPAR γ
PPAR α
LXR L
LXR
IFN- γ -induced gene expression,
e.g. MCP-1

7.3 Future work

A number of avenues for further investigation are highlighted by the results of this study. These can be divided into investigation of the general roles of ERK and JNK, along with other potential kinases in the regulation of gene expression and cellular responses by IFN-γ, and the continued in depth analysis of the mechanisms involved in the inhibitory effects of the therapeutic agents on IFN-γ signalling.

7.3.1 Future investigations into the roles of ERK and JNK in the regulation of gene expression by IFN-γ

The initial step in the continued investigation of the roles of ERK and JNK along with other kinases in the regulation of IFN-γ-mediated gene expression in macrophages is to extend the studies by other approaches such as siRNA/shRNA-mediated knockdown. It is also of great interest to confirm some of our findings on cells from ERK-, JNK- or other kinase-deficient mice.

To investigate the potential role of ERK5-dependent pathway in the IFN-γ-mediated STAT1 transcriptional activity and gene expression, it would be necessary to extend the studies via the use of DN approaches used for studies in this thesis or by specific knock-down of ERK5 or its upstream kinase MEK5 with siRNA/shRNA. The phosphorylation state and kinase activity of ERK5 in response to the cytokine would also need to be monitored.

The role of Ser727 in the regulation of STAT1 activity is at present unclear but appears to be associated with the recruitment of co-factors such as MCM5, BRCA1 and CBP/p300 (Zhang et al., 1998; Ouchi et al., 2000; Sun et al., 2005). Chromatin immunoprecipitation (ChIP) assays is an elegant technique that allows the determination of which co-factors are involved in the transcriptional regulation of a certain gene (e.g. MCP-1). Briefly, ChIP involves: the cross-linkage of proteins, including regulatory factors, to chromatin using formaldehyde; chromatin

fragmentation; immunoprecipitation of the protein of interest; reversal of cross-linking; followed by PCR analysis using primers specific for the section of promoter region of interest. The results of these investigations would verify whether these sequences bind to co-regulator proteins and reveal any potential gene-specific differences. Recruitment of CBP/p300 to STAT1 target gene promoters in response to IFN-γ has previously been demonstrated by Sun et al. (2005) using this technique. ChIP assays have also been employed in our laboratory to determine the binding of STAT1 and Sp1 to the MCP-1 gene promoter (Harvey, 2006). It thus could be used to determine the recruitment of STAT1 and specific co-regulators on the promoter of genes that have been identified to be regulated through ERK and JNK pathways. The effect of blockage of these two pathways on the recruitment of these factors could also be investigated. Blockage of ERK and JNK could be achieved by pharmacological inhibitors, DN mutants, siRNA or the use of cells derived from ERK- or JNK-knockout mice.

As discussed earlier, although it has been clearly shown that ERK and JNK mediate the regulation of IFN-γ responsive gene expression, at least in part, through the modulation of STAT1 phosphorylation in human macrophages, we do not rule out the possibility that these two kinases may also regulate gene expression through alternative STAT1-independent pathways. For example, STAT3 has been shown to replace STAT1 in STAT1-null cells to drive the transcription of certain genes, such as SOCS-3 and CEBPδ in response to IFN-γ (Qing and Stark, 2004; Ramana et al., 2005). Amongst the genes analysed in this study, the expression of MCP-1 and MIP-1β has been shown to be induced by IFN-γ in both wild type and STAT1-null macrophages (Gil et al., 2001). The study therefore could initially extend to investigation of the role of STAT3 in the regulation of MCP-1 and MIP-1β expression in the STAT1-null cells. STAT3 phosphorylation and the effect of STAT3-knockdown by siRNA on gene expression in STAT1-null cells might be two of the potential approaches for future study. The effect of blockage of ERK and JNK on the potential

STAT3 transcriptional activity could then be investigated.

Cross-talk between different signalling pathways is relatively common in the regulation of gene expression in response to IFN-γ as discussed in Chapter 4 (section 4.4.4). It is thus important that further studies are carried out in relation to the potential cross-talk between the ERK/JNK pathways and others, such as CK2, PI3K, PKCδ and CaMKII pathways. Such studies could involve analysis of the activation of a particular pathway (e.g. ERK) in cells where the action of key components of other signalling pathways has been inhibited either by the use of pharmacological agents, siRNA-mediated knockdown or use of macrophages from knockout mice.

Adhesion assays conducted in this study have shown that the inhibitors for ERK, JNK and PI3K prevent the IFN-γ-stimulated adhesion of monocytes to endothelial cells. It is, however, difficult to come to any firm conclusions on the exact roles of these kinases in the adhesion process due to the reduced viability of endothelial cells as a result of incubation with the inhibitors for a prolonged period of time. It is thus of particular importance to further confirm the results through alternative approaches. SiRNA/shRNA-mediated knockdown of each kinase might represent one potential approach instead of the use of pharmacological inhibitors.

7.3.2 Future investigation into the mechanisms underlying the inhibitory effects of the therapeutic agents on IFN-γ signalling

Although most of the actions of PPAR or LXR agonists are mediated through the corresponding NR, we have noticed that a number of previous studies on transrepression by these agonists on IFN-γ-mediated gene expression have suggested some NR-independent effects (see Table 1.7 for a list of studies). In addition, it remains to be determined what the exact role of RXR is in the 22R/9cRA-mediated inhibition of IFN-γ responses as discussed above. It is therefore essential that this is investigated before detailed studies on the mechanisms underlying transrepression are carried out. This could be achieved through the analysis of the actions of the agonists on mRNA expression of marker genes and/or STAT1 phosphorylation/transactivation in cells where the expression of the corresponding NR has been knocked down by RNAi approaches.

EMSA analysis has shown that the activation of LXRs, but not PPARs or simvastatin inhibits the STAT1 DNA binding *in vitro*. It will therefore be of interest to confirm whether this is also the case *in vivo* using ChIP assays. Such analysis could then be extended to a range of genes, in order to discriminate between potentially global effects and those that are likely to be promoter-specific.

Initial studies on the nucleo-cytoplasmic translocation using western blot analysis suggest that activation of LXR inhibits the IFN-γ-induced nuclear accumulation of activated phospho Tyr701 STAT. LXR agonists have also been shown to attenuate the STAT1 phosphorylation at Tyr701 site. The possibility therefore remains that the reduced nuclear amounts of Tyr701 phospho-STAT1 observed is merely due to inhibition of STAT1 phosphorylation. To further confirm the effect of these potential therapeutic agents on nuclear accumulation of STAT1, immunoflorescence analysis should be carried out as used in numerous publications studying the nucleo-cytoplasmic cycling of STAT1 (Begitt et al., 2000; Meyer et al., 2003; Sadzak

I et al., 2008). For this, the cells are pre-treated with the corresponding agent and subsequently stimulated with IFN-γ for the appropriate period of time (the optimal time point should be determined from initial experiments). STAT1 in fixed cells could then be visualised by indirect immunofloresence microscopy using an anti-STAT1 antibody and fluorescently-labelled secondary antibody [e.g. Cy3-labelled goat anti-rabbit antibody (Jackson ImmunoResearch, Cambridge, UK)]. Confocal microscopy available in our university is a powerful tool that could produce images with high resolution to study the location of the protein and dynamic processes in the cells.

Both PPAR-γ and LXRs are subjected to SUMOylation. Such SUMOylation and NCoR-dependent mechanism has been found to be responsible for transrepression of LPS-induced expression of certain genes in mouse macrophages (Pascual et al., 2005; Ghisletti et al., 2007). It is thus of particular interest to probe whether this is also important for transrepression of IFN-γ signalling. The study could involve investigating the effect of RNAi-mediated knockdown of the rate-limiting, E2-conjugating enzyme Ubc9, that is required for SUMOylation of target proteins (Hay, 2005). The read-out could mainly be changes in mRNA expression of key downstream targets by PCR analysis. To further test the ligand-dependent SUMOylation of NRs, both *in vitro* and *in vivo* approaches could be used. *In vitro* SUMOylation reactions could be carried out by adding recombinant SUMO and Ubc9 to *in vitro*-translated NRs [see (Ghisletti et al., 2007) for details]. The NRs can be synthesized *in vitro* directly from PCR products using commercially available *in vitro* translation kits, such as PROTEINscript™ II (Ambion/Applied Biosystems, Warrington, UK). For *in vivo* SUMOylation assays, the plasmids specifying for FLAG-tagged NR is transfected into cells in the absence or presence of those for Myc-tagged SUMO-1, -2 or -3. Protein extracts are subjected to western blot analysis using anti-FLAG antibodies where the appearance of higher molecular weight is indicative of potential SUMOylation, and the presence of SUMO moiety can be

confirmed by western blot using anti-Myc antibodies (see (Pascual et al., 2005; Ghisletti et al., 2007) for details).

Ant-inflammatory effects of statins have been suggested to be mediated through PPAR-dependent pathways (Paumelle and Staels, 2007). In order to investigate the importance of each PPAR isoform in statins-dependent inhibition of IFN-γ-induced gene expression, RNAi-mediated knockdown of corresponding PPAR isoforms could be carried out. The changes in statins-mediated inhibition of the IFN-γ response marker genes could be then monitored using PCR. It would also be of interest to evaluate whether there is a synergistic action of statins and PPAR ligands on IFN-γ signalling. This could be achieved by investigating the IFN-γ-induced gene expression, STAT1 phosphorylaiton or transactivation in response to statins and PPAR ligands individually and in combination.

A recent study suggested that statins inhibit the CRP-induced inflammatory gene expression by attenuating the ERK pathway (Montecucco et al., 2009). In order to evaluate whether this is also the case in IFN-γ signalling, the study could be initially extended to investigate the effect of statins on IFN-γ-induced ERK activity. The effect of knockdown of ERK (e.g. by RNAi) on statins-mediated inhibition of gene expression could also been studied mainly using RT-PCR analysis.

7.4 Implications for the treatment of atherosclerosis

Recent studies have demonstrated the selective targeting of protein kinases as a therapeutic approach, and it is a common procedure to use small inhibitor molecules (Daub et al., 2004; Buschbeck, 2006; Roberts and Der, 2007). Indeed, potential therapeutic strategies have been investigated in relation to the treatment of inflammatory diseases that involve the inhibition of ERK- or JNK-dependent signalling (Bennett, 2006; Schuh and Pahl, 2009). Further research into these pathways could lead to the development of specific therapeutics without undesired side effect. The identification of the molecular mechanisms underlying the anti-inflammatory effects of agonists for PPARs and LXRs, as well as statins, in relation to IFN-γ responses provides additional targets for therapeutic intervention.

Chemokines and adhesion molecules are key inflammatory mediators that play pivotal roles in the recruitment of inflammatory cells to lesion sites, a crucial early step in atherogenesis (section 1.4). Previous studies have demonstrated that the deficiency of several chemokines or adhesion molecules in mouse models of atherosclerosis protects against lesion formation (Collins et al., 2000; Cybulsky et al., 2001; Ni et al., 2001; Inoue et al., 2002b). The broad-spectrum inhibitor of chemokines has also been investigated for a potential treatment of inflammatory diseases with some success (Grainger and Reckless, 2003; Johnson et al., 2004). The identification of ERK-dependent pathways in the regulation of chemokine and adhesion molecule expression by IFN-γ in macrophages suggests a beneficial, athero-protective effect of specific inhibition of ERK signalling. The suggestion of potential role for ERK in the IFN-γ-induced adhesion of monocytes to endothelial cells adds further support to the potential therapeutic significance of this signalling pathway for atherosclerosis. The inhibition of ERK activity by specific pharmacological inhibitors has previously been suggested as a potential therapeutic strategy in pulmonary inflammatory disease (Duan et al., 2004; Schuh and Pahl, 2009). Investigation into therapeutic strategies for

treatments of cancer that involves the inhibition of ERK-dependent signalling have led to great success and have been taken into phase I or II clinical trials (Rinehart et al., 2004; LoRusso et al., 2005; Roberts and Der, 2007; Adjei et al., 2008). It is possible that these may be applied to atherosclerosis.

The requirement of JNK in IFN-γ responses suggests that signalling through this kinase may also provide a target for therapeutic intervention of atherosclerosis. SP600125 has been used to attenuate disease progression in a number of rodent models of pulmonary inflammatory disorders, including single and chronic allergen challenge in rats (Eynott et al., 2003; Eynott et al., 2004) and mouse (Nath et al., 2005). Based on the numerous findings on crucial roles of JNK in immune and inflammatory responses, it has been assumed that JNK inhibitors might reduce leukocyte infiltration as well as the expression of chemokines in pulmonary cells (Bennett, 2006), consistent with our findings in macrophages in this study. The therapeutic strategy based on the use of low molecular weight compounds, such as SP600125, may thus also be applicable to atherosclerosis.

The findings in this study clearly demonstrated that the agonists for PPAR-α and -γ, as well as statins, inhibit the IFN-γ-induced chemokine and CIITA gene expression. The results add direct support to the crucial anti-inflammatory and possibly immono-modulatory actions of these therapeutic agents and indicate the potential therapeutic benefits for these agents for the treatment of atherosclerosis, and possibly other inflammatory diseases. Although the underlying mechanisms are at present unclear, compelling evidence from animal and clinical studies has indicated that PPARα (fibrates) and PPARγ (TZD) could exert cardio-protective effects through their anti-inflammatory actions (Sidhu et al., 2003; Yue et al., 2003; Wang et al., 2005; Yu et al., 2007; Bulhak et al., 2009). The potentially beneficial pleiotropic effects of statins on atherosclerosis are undergoing a number of clinical trials and preliminary studies have generated promising results (Davignon and Leiter, 2005).

Despite the potentially important therapeutic benefits of these agents, these drugs possess a number of deleterious side effects, which have not only restrained their clinical use but have also led to the unsuccessful clinical development of many PPAR agonists (Rubenstrunk et al., 2007; Shearer and Billin, 2007). Therefore, one of the excellent strategies for the development of PPAR agonists-derived drugs for the treatment of atherosclerosis would be to preferentially transrepress inflammatory genes in key atheroma cells, instead of transactivating detrimental metabolic genes, thereby retaining their athero-protective actions without undesired side effects. Our findings on the anti-inflammatory actions of these therapeutic agents in relation to IFN-γ responses in macrophages suggests that inhibition of IFN-γ-induced pro-inflammatory gene expression provide potentially valuable therapeutic target for the development of next generation of cell-type- and target gene-type-selective PPAR agonists. A newly synthesised PPARγ ligand MBX-102/JNJ39659100 with selective activity has shed light on the development of such therapeutic drugs. This new type of ligand has been shown to act as a selective PPARγ activator with weak transactivation but robust transrepression activity and therefore exhibits full therapeutic activity without the classical PPARγ side effects (Gregoire et al., 2009). Likewise, the development of cell-type- or tissue-selective statins could potentially be a better therapeutic approach without the undesired side effects.

The inhibitory effects of LXR ligands on the IFN-γ-mediated responses provide further evidence that these agonists could protect against the development of atherosclerosis, not only due to their well-appreciated roles in promoting reverse cholesterol transport but also their potential function in the regulation of immune and inflammatory responses. LXR ligands therefore may be valuable candidates for the development of novel therapeutic approaches for the treatment of inflammatory conditions beyond atherosclerosis. Indeed, the potential therapeutic benefits of LXR ligands for the treatment of pulmonary inflammation have been previously demonstrated in cultured macrophages and mouse models *in vivo* (Birrell et al., 2007;

Smoak et al., 2008).

The activation by the LXRs of *de novo* lipogenesis and associated elevation of plasma VLDL-triglyceride levels represents a major challenge for pharmaceutical development of ideal LXR agonists for the treatment of atherosclerosis (Grefhorst et al., 2002). One of the potential strategies to minimise the side effects of LXR ligands is the identification of target gene-selective or tissue-selective LXR agonists. Our results clearly demonstrate the inhibitory effects of LXR ligands on IFN-γ signalling, via targeting STAT1-dependent pathway in macrophages, thereby suggesting that STAT1-mediated IFN-γ signalling in macrophages provides specific targets for the development of novel therapeutic approaches.

7.5 Concluding remarks

From the work presented in this thesis, it is clear that IFN-γ mediates the regulation of STAT1-dependent pathway in macrophages via several signalling cascades. The IFN-γ-dependent signalling and the target genes studied have important functions in the pathogenesis of atherosclerosis. Moreover, the studies presented in this thesis also identified crucial anti-inflammatory actions of agonists for PPARs and LXRs, as well as statins on IFN-γ-mediated responses in macrophages. A complete understanding of the molecular mechanisms involved in the key signalling pathways and the anti-inflammatory effects of these therapeutic agents will hopefully lead to the development of new therapeutic strategies to combat this disease.

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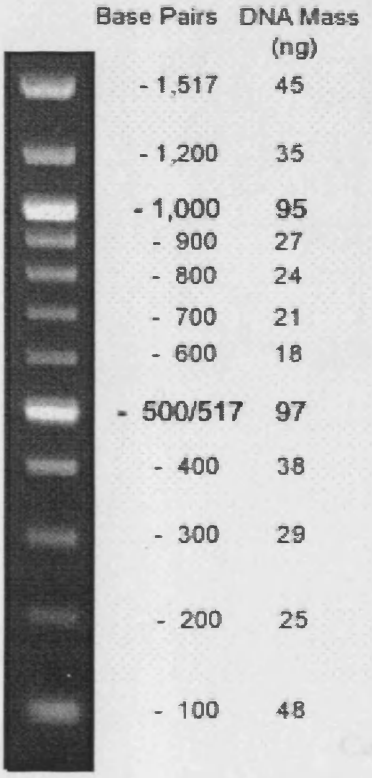
APPENDICES

Appendix I

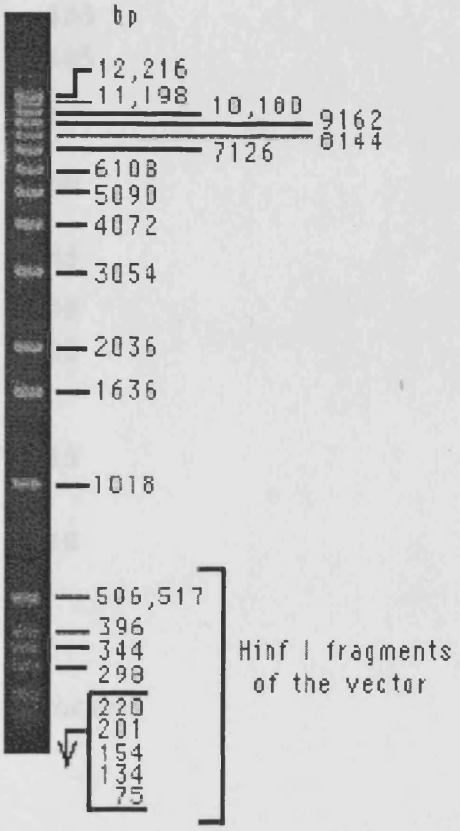
DNA molecular weight markers

NEB 100bp ladder



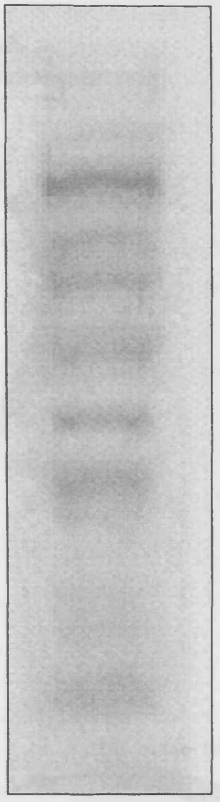
1.3% agarose gel
0.5µg/lane
Cat No. N3231S/N3231L

Invitrogen 1kb ladder



0.9% agarose gel
0.5µg/lane
Cat No. 15615-016

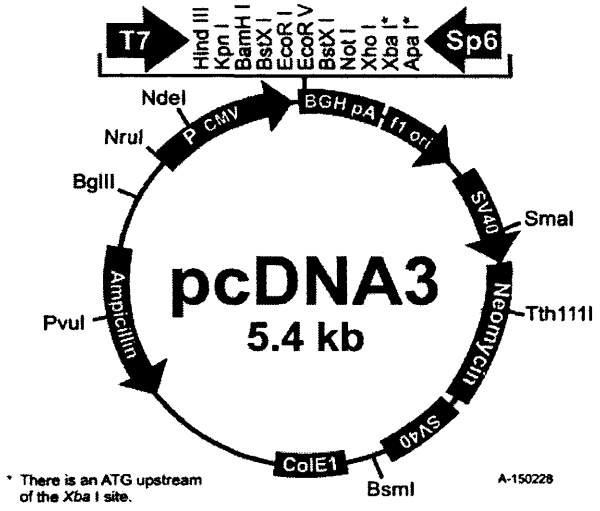
Full-range Ranbow™ Molecular Weight Markers

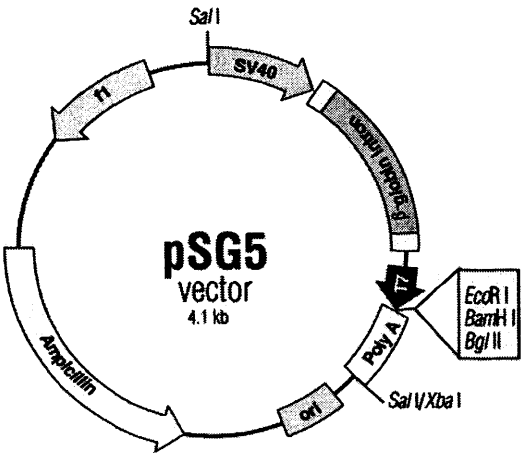


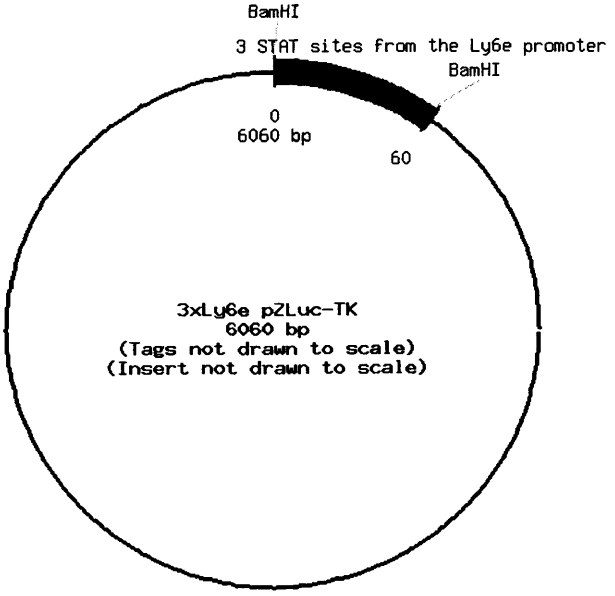
12% SDS-PAGE gel
Cat. No. RPN800 (GE healthcare)

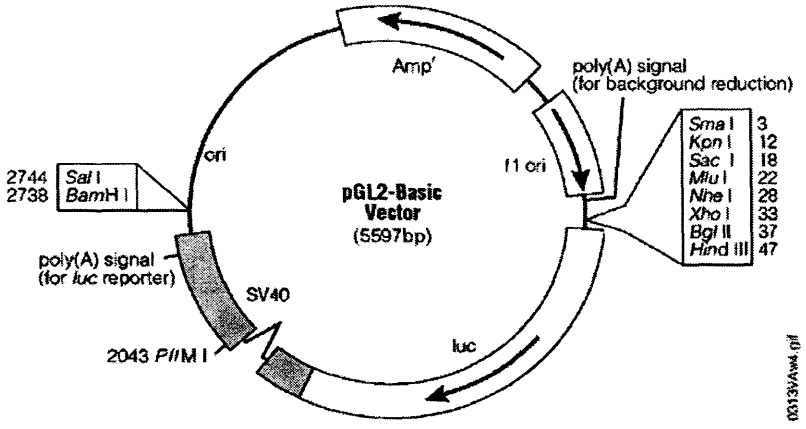
Appendix II

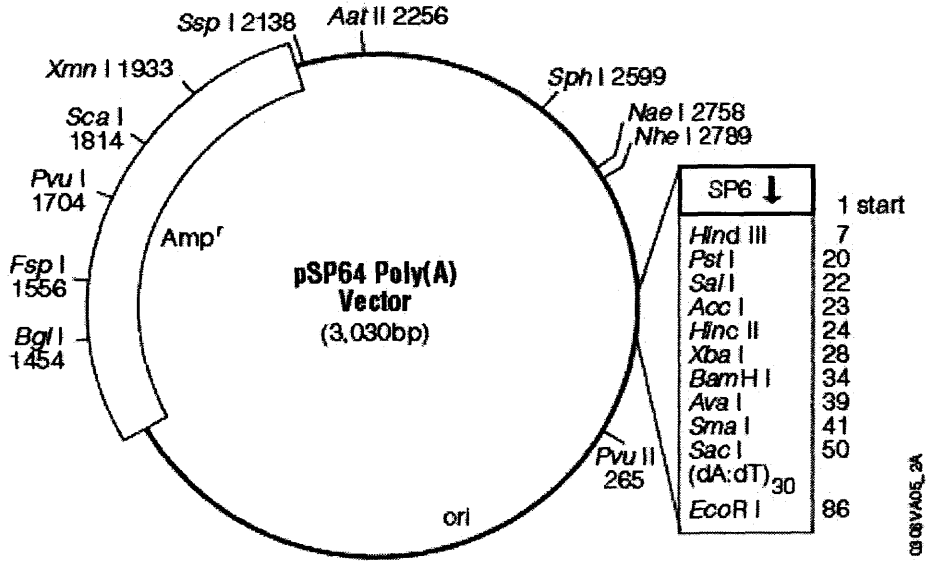
Plasmid vectors

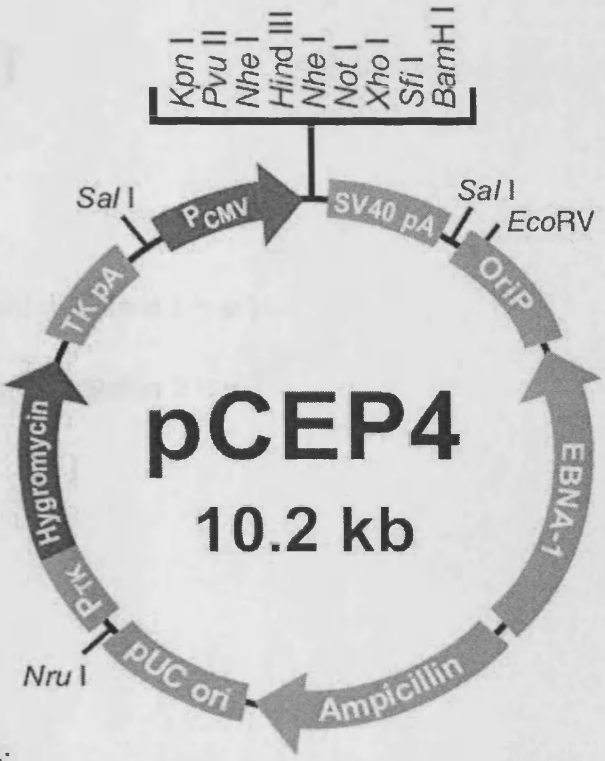




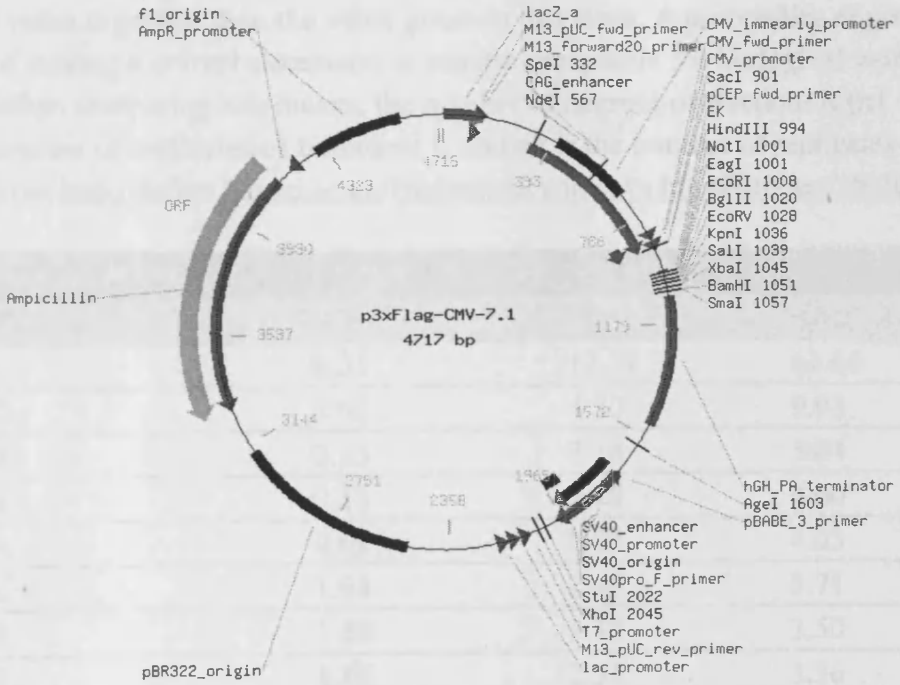



Amp'
poly(A) signal
(for background reduction)
Sma I 3
Kpn I 12
Sac I 18
Mlu I 22
Nhe I 28
Xho I 33
Bgl II 37
Hind III 47
ori
f1 ori
2744 Sal I
2738 BamH I
pGL2-Basic
Vector
(5597bp)
poly(A) signal
(for luc reporter)
SV40
2043 PflM I
luc


Ssp I 2138
Aat II 2256
Xmn I 1933
Sca I
1814
Pvu I
1704
Fsp I
1556
Bgl I
1454
Amp'
pSP64 Poly(A)
Vector
(3,030bp)
ori
Sph I 2599
Nae I 2758
Nhe I 2789
Pvu II
265
SP6
1 start
Hind III 7
Pst I 20
Sal I 22
Acc I 23
Hinc II 24
Xba I 28
BamH I 34
Ava I 39
Sma I 41
Sac I 50
(dA:dT)30
EcoR I 86



Comments for pCEP4:
10186 nucleotides



Appendix III

t-Test

Mean 1 = $\bar{x}_1$ Standard deviation 1 = σ_1

Mean 2 = $\bar{x}_2$ Standard deviation 2 = σ_2

Variance = σd^2

$$\sigma_d^2 = \frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sigma_d}$$

t-Table

A difference between two means is significant (at the given probability level) if the calculated t value is greater than the value given in this table. A probability of p = 0.05 (95% probability of making a correct statement) is usually acceptable for biological work.
When comparing two means, the number of degrees of freedom is (n1 + n2)-2, where n1 is the number of replicates of treatment 1, and n2 is the number of replicates of treatment 2 (adapted from http://helios.bto.ed.ac.uk/bto/statistics/tress4a.html#Student's%20t-test).

Degrees of Freedom	t-Value			
P-Value	0.1	0.05	0.01	0.001
1	6.31	12.71	63.66	636.62
2	2.92	4.30	9.93	31.60
3	2.35	3.18	5.84	12.92
4	2.13	2.78	4.60	8.61
5	2.02	2.57	4.03	6.87
6	1.94	2.45	3.71	5.96
7	1.89	2.37	3.50	5.41
8	1.86	2.31	3.36	5.04
9	1.83	2.26	3.25	4.78
10	1.81	2.23	3.17	4.59

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