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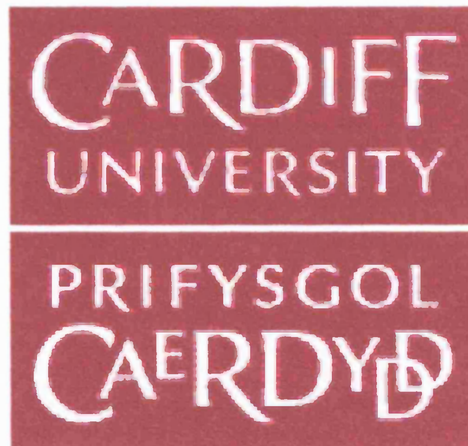

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A thesis presented for the degree of Doctor of Philosophy

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March 2006

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ABSTRACT

The cytokine IFN-γ regulates the expression of numerous genes in macrophages, including many that are implicated in atherosclerotic processes. Studying the signalling mechanisms involved in the mediation of IFN-γ responses may lead to the identification of future therapeutic targets for the treatment of atherosclerosis.

Previous work in our laboratory revealed a role for the kinase CK2 in modulating the expression of the LPL and ICER genes in response to IFN-γ. Experiments detailed in this report show that the CK2 inhibitor apigenin inhibited the IFN-γ-mediated induction of each gene in the group selected for study (ICER, MCP-1, SOCS-1, IP-10, Mig, I-TAC, CCR2), suggesting that there may be a common role for CK2 in the regulation of macrophage gene expression by the cytokine. The PI3K inhibitor LY294002 has also previously been found in our laboratory to prevent the IFN-γ-mediated suppression of LPL expression. In this study LY294002 selectively inhibited the induction of MCP-1, IP-10, Mig, I-TAC, and CCR2 expression by IFN-γ but not that of ICER or SOCS-1. Extension of this line of investigation through microarray analysis provided further evidence for a gene-specific requirement for PI3K in mediating IFN-γ responses.

The signalling pathways involved in the regulation of MCP-1 and ICER gene expression by IFN-γ were chosen for more detailed study. The JAK-STAT pathway is the most widely accepted mechanism of IFN-γ signalling. However, recent studies have suggested the existence of potential STAT1-independent pathways. The functions of CK2 and PI3K were investigated, in relation to a STAT1-dependent or -independent mechanism of IFN-γ signalling, in the regulation of MCP-1 and ICER expression.

A role for the JAK-STAT pathway in the regulation of MCP-1 expression by IFN-γ was revealed, in macrophages, through co-transfection studies with inactive mutant forms of JAK1, JAK2 and STAT1. Promoter binding analyses demonstrated that IFN-γ induced the binding of STAT1 to a GAS site in the MCP-1 promoter. PKB was found to act as a downstream effector for PI3K in the IFN-γ-mediated induction of MCP-1. Both CK2 and PI3K/PKB were found to be involved in the IFN-γ-mediated activation of STAT1 through phosphorylation at Serine 727. Additionally, constitutive Sp1 protein binding to the MCP-1 promoter was demonstrated. Co-immunoprecipitation revealed an IFN-γ-inducible interaction between Sp1 and STAT1 that may function in the cytokine response.

In our laboratory CK2 has previously been shown to phosphorylate CREB in response to IFN-γ. A luciferase reporter construct containing four tandem CRE consensus elements, used to model the ICER promoter that contains four CRE-like sites, showed inducible activation by IFN-γ, through CK2. The JAK2 inhibitor AG490, and dominant negative forms of JAK1, JAK2 and STAT1, did not attenuate increases in ICER expression in response to IFN-γ. These findings indicate that CREs represent a novel class of IFN-γ-responsive element and that the regulation of ICER expression may occur through CK2 in a JAK-STAT-independent pathway.

Together these studies have demonstrated novel roles for the kinases CK2 and PI3K in the regulation of macrophage gene expression by IFN-γ.

ABBREVIATIONS

ABBREVIATION	FULL TERM
15-LO	15-lipoxygenase
ABCA1	ATP-binding cassette transporter A1
ACAT	Acyl coenzyme A:acylcholesterol transferase
AMPPNP	5'-[β,γ-imido]triphosphate
AP-1	Activator protein-1
ApoE	Apolipoprotein E
APRIL	Apoptosis-inducing ligand
ATF	Activating transcription factor
BAFF	B-cell activating factor belonging to the TNF family
BCA-1	B lymphocyte chemoattractant-1
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BRCA1	Breast cancer susceptibility gene 1
bZIP	Basic-leucine zipper
C/EBP	CAAT-enhancer binding protein
C3G	Crk-SH3-binding GEF
cAMP	Cyclic AMP adenosine monophosphate
CARE	cAMP autoregulatory element
CBP	CREB-binding protein
c-Cbl	Cancer-causing-Casitas-B-lineage lymphoma
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD36	Complementarity determinant 36
CD40	Complementarity determinant 40
CD44	Complementarity determinant 44
ChIP	Chromatin immunoprecipitation
CIITA	Class II transactivator
CK2	Casein kinase 2
CNAP	Condensation-related SMC-associated protein
CNT	Concentrative nucleoside transporter
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CREM	cAMP-responsive element modulator protein
CRP	C-reactive protein
CTACK	Cutaneous T-cell-attracting chemokine
CX3CL	CX3C-chemokine ligand
CX3CR	CX3C-chemokine receptor
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
EGF	Epidermal growth factor
eIF	Elongation initiation factor
ENA-78	Epithelial neutrophil-activating peptide 78
eNOS	Endothelial NOS
ENT	Equilibrative nucleoside transporter
ERK	Extracellular signal related kinase
E-selectin	Endothelial selectin
FAH	Fumarylacetoacetate hydrolase
FGF	Fibroblast growth factor
FKBP-12	FK506-binding protein-12
FKRD	Forkhead transcription factor
FSH	Follicle-stimulating hormone
Fyn	Src-yes-related novel protein tyrosine kinase
GAPDH	Glyceraldehyde phosphate dehydrogenase
GARG	Glucocorticoid attenuated response gene
GAS	Gamma-activated sequence
GATE	Gamma-activated transcriptional element
GBF	GATE-binding factor

GBP	Guanylate-binding protein
GCP	Granulocyte chemotactic protein
GH	Growth hormone
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRF	Gamma-responsive factor
GRO	Growth-related oncogene
GSK-3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
HB-EGF	Heparin binding epidermal growth factor
HCC	Human CC chemokine
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
IκB	Inhibitor of κB
ICAM	Intercellular adhesion molecule
ICE	IL-1-converting enzyme
ICER	Inducible cAMP early repressor
ICH-3	ICE/Ced-3 family
IFN	Interferon
IFN-γR	IFN-γ-receptor
IGF	Insulin-like growth factor
IGTP	Inducibly expressed GTPase
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible NOS
IP-10	Interferon inducible protein-10
IRF	Interferon regulatory factor
IRG	G-protein induced by IFN
IRS	Insulin receptor substrate
ISG	IFN-stimulated gene
ISRE	Interferon-stimulated response element
I-TAC	Interferon-inducible T-cell alpha chemoattractant
JAK	Janus kinase
JNK	c-Jun amino-terminal protein kinase
LAP	Liver-enriched activating protein
LDL	Low density lipoprotein
LDL-R	LDL-receptor
LIF	Leukaemia inhibitor factor
LIGHT	Homologous to lymphotoxins, inducible expression, competes with herpes simplex virus glycoprotein D for herpes virus entry mediator (HVEM/TR2)
LIP	Liver-enriched inhibitor protein
LMP-1	Latent membrane protein-1
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRG	G-protein induced by LPS and IFN
LRP	LDL-receptor related protein
LT	Lymphotoxin
MAPK	Mitogen activated protein kinase
MCM5	Mini chromosomal maintenance protein 5
MCMV	Murine cytomegalovirus
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage-colony stimulating factor
MDA-7	Melanoma-differentiation associated gene-7
MDC	Macrophage-derived chemokine
MEC	Mammary-enriched chemokine
Mel-N1	Neuronal RNA-binding protein
MgluR8	Metabotropic glutamate receptor
MHC	Major histocompatibility complex
Mig	Monokine Induced by IFN-γ
MIP	Macrophage inflammatory protein
MKP-1	MAP kinase phosphatase 1
MLK	Mixed lineage kinase

mmLDL	Minimally modified LDL
MMP	Matrix metalloproteinase
MPIF-1	Myeloid progenitor inhibitory factor-1
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NAP-2	Neutrophil-activating peptide 2
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NF-YA	Nuclear factor Y-subunit
NGF	Neuronal growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase
oxLDL	Oxidised LDL
PDGF	Platelet-derived growth factor
PF4	Platelet factor 4
PH	Pleckstrin homology (domain)
PI	Phosphatidyl-inositol
PI3K	Phosphoninositide 3-kinase
PIP	Phosphatidyl-inositol phosphate
pIRE	Palindromic interferon response element
PKA	Protein kinase A
PKB	Protein kinase B (Akt)
PKC	Protein kinase C
PKR	dsRNA-dependent protein kinase
PRL	Prolactin
PRP	Precursor RNA processing protein
P-selectin	Platelet selectin
pTEFb	Positive transcription elongation factor-b
PTEN	Phosphatase and tensin homolog
Pyk2	Protein tyrosine kinase 2
Rac	Ras-related C3-botulinum toxin substrate
Raf	Rous sarcoma associated factor
RANK	Receptor activator of NF-κB
RANTES	Regulated upon activation, normal T cell expressed and secreted
Rap	Receptor associated protein
Rb	Retinoblastoma protein
Ro RNP	Ribonucleoprotein
ROS	Reactive oxygen species
S6K	Ribosomal protein S6 kinase (p70)
SCM-1β	Single cysteine motif-1β
SDF-1	Stromal-cell-derived factor 1
SH2	Src homology 2 (domain)
SHIP	SH2-containing inositol 5' phosphatase
SHP	SH2-binding protein
SLC	Secondary lymphoid tissue chemokine
SOCS	Suppressor of cytokine signalling
SOD	Superoxide dismutase
Sp	Specificity protein
SR	Scavenger receptor
SREBP	Sterol-regulatory element binding protein
SSRE	Shear stress-regulatory element
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokine
TBP	TATA-binding protein
TDAG-51	Pro-glu-his rich protein
TECK	Thymus-expressed chemokine
TGF	Transforming growth factor
TGIF	5'-TG-3'-interacting factor
TGTP	T-cell GTP-binding protein
TI-227	Cancer metastasis associated gene
TNF	Tumor necrosis factor

TPA	Tumor promoting agent
TPL-2	Tumor promotion locus-2
TRAIL	TNF-α related apoptosis inducing ligand
TRE	TPA responsive element
TSH	Thyroid stimulated hormone
Tyk	Tyrosine kinase
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelium derived growth factor
VLDL	Very low density lipoprotein
XKLF	Kruppel-like factor
YB-1	Y-box binding protein-1
Zfx	Zinc finger protein

Reagents, units and general scientific terminology

ABBREVIATION	FULL TERM
APS	Ammonium persulphate
ATP	Adenosine triphosphate
b	Base
BMM	Bone marrow-derived macrophage
bp	Base pair
BSA	Bovine serum albumin
C	Carboxyl (terminus)
CAD	Coronary artery disease
cDNA	Copy DNA
CMV	Cytomegalovirus
cRNA	Copy RNA
Da	Dalton
DBD	DNA-binding domain
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DN	Dominant negative
DNA	Deoxyribonucelic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EC	Endothelial cell
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
g	Grams
g	Gravity
gDNA	Genomic DNA
GTP	Guanidine triphosphate
h	Hours
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HI-FCS	Heat inactivated-fetal calf serum
HUVEC	Human umbilical vein endothelial cell
IP	Immunoprecipitation
IUIS/WHO	International Union of Immunological Societies/World Health Organisation
k	Kilo
log	Logarithm
Luc	Luciferase
M	Molar
m	Metres
MEF	Mouse embryonic fibroblast

min	Minutes
MMLV	Molony murine leukaemia virus
mRNA	Memory RNA
N	Amino (terminus)
NA	No antibody
NK	Natural killer (cell)
NKT	Natural killer T (cell)
Pa	Pascals
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pen/strep	Penicillin/streptomycin
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulphonyl fluoride
Pol	Polymerase
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RSV	Rous sarcoma virus
RT	Reverse transcriptase
s	Seconds
SD	Standard deviation
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SMC	Smooth muscle cell
SV40	Simian virus 40
T	Treated
TBE	Tris-borate-ethylenediaminetetracetic acid
TE	Tris-ethylenediaminetetracetic acid
TEMED	N, N, N',N, tetramethylenediamine
TF	Transcription factor
Th	T-helper (cell)
tRNA	Transfer RNA
U	Units
UT	Untreated
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volts
v/v	Volume/volume
VSMC	Vascular smooth muscle cell
w/v	Weight/volume

Amino acids

AMINO ACID	THREE-LETTER CODE	SINGLE-LETTER CODE
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chemical elements

ELEMENT	SYMBOL
Calcium	Ca
Carbon	C
Chlorine	Cl
Fluorine	F
Hydrogen	H
Magnesium	Mg
Nitrogen	N
Oxygen	O
Phosphorus	P
Potassium	K
Sodium	Na
Sulphur	S
Vanadium	V
Zinc	Zn

Chapter one:

Introduction

CHAPTER 1. INTRODUCTION

1.1 INTRODUCTION

The rising incidence of obesity in the Western world is accompanied by an increase in the proportion of the population affected by coronary artery disease (CAD). There is therefore an ever increasing demand for new therapies for the combat of coronary conditions. Atherosclerosis is a primary cause of CAD, characterised by the formation of a lipid-filled plaque within the wall of large arteries. One of the major risk factors for the disease is the presence of high levels of cholesterol in the blood. 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). However there is still a need for new and more effective treatments for atherosclerosis.

The development of the atherosclerotic lesion is a chronic inflammatory process and is governed by a complex network of signalling pathways. Although statins may have some antiinflammatory effects, the therapeutic strategies currently in place largely ignore the inflammatory component of the disease (Lefer 2002). 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 focus of this thesis is the mechanisms by which the proinflammatory cytokine interferon (IFN)-γ regulates gene expression. In particular, the role of IFN-γ in inducing the expression of the proatherogenic chemokine monocyte chemoattractant protein (MCP)-1 was studied in detail.

1.2 CYTOKINES

Cytokines are small, regulatory proteins (most below 30kDa in size) with pleiotropic actions, particularly in the regulation of the immune system and the inflammatory response. White blood cells secrete numerous cytokines although they are also produced by a variety of other cells in the body. Constitutive expression of cytokines is usually low, a range of stimuli inducing transient production. Typically cytokines act over short distances by binding to specific cell surface receptors and causing changes in the pattern of gene expression. Cytokines exert a multitude of actions on a variety of cells, including regulation of cell proliferation, changes in the state of differentiation, chemotaxis and other effects on the inflammatory response (Vilcek 2003).

It is possible to group cytokines into families on the basis of their structural features. Families include: interleukins (IL-1 to IL-26); interferons (*e.g.* IFN-α, IFN-β, IFN-ω and 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-β, bone morphogenetic proteins (BMPs)); and other growth factors (*e.g.* platelet-derived growth factor (PDGF), epidermal growth factor (EGF)) (Table 1.1) (Vilcek 2003).

There is a high degree of functional redundancy among the different cytokines and cytokines that are structurally very distinct often exhibit similar effects (*e.g.* TNFα and IL-1 (Zhao *et al.* 2003, Le and Vilcek 1987)). There are also many examples of cytokines acting synergistically (*e.g.* TNF-α and IFN-γ in the regulation of lipoprotein lipase (LPL) (Tengku-Muhammad *et al.* 1998) or ICAM-1 expression (Jahnke and Johnson 1994); IL-18 with IL-2 or IL-12 in the induction of Th1/Th2 cytokine expression (Rodriguez-Galan 2005)) or antagonistically (*e.g.* IL-4 and IFN-γ in the regulation of iNOS expression (Jungi *et al.* 1997), or collagen biosynthesis (Sempowski *et al.* 1996); IL-1 and TGF-β observed over genome wide gene expression analysis (Takahashi *et al.* 2005a)). In the inflamed tissue a large number of cytokines are present and the network of interactions between them is likely to be extremely complex.

Table 1.1 Division of cytokines into families based on structural features

FAMILY	REPRESENTATIVE MEMBERS
Chemokines	CC subfamily (CCL1-28) CXC subfamily (CXCL1-16) C subfamily (XCL1-2) CX3C subfamily (CX3CL1)
IL-1	IL-1α IL-1β IL-1 receptor antagonist IL-18
IL-10	IL-10 IL-19 IL-20 IL-22 IL-24 (MDA-7)
IL-17	IL-17 IL-25
IL-2/IL-4	IL-2 IL-4 IL-5 GM-CSF
IL-6/IL-12	IL-6 IL-12
IFNs	IFN-α IFN-β IFN-ε IFN-κ IFN-ω IFN-γ IFN-τ IFN-λ(1-3)
TGF-β	TGF-β BMPs Inhibins Activins
Tumor necrosis factors	TNF-α LT-α (TNF-β) LT-β Fas ligand CD40 ligand TRAIL BAFF APRIL RANK LIGHT

Abbreviations: APRIL, apoptosis-inducing ligand; BAFF, B-cell activating factor belonging to the TNF family; BMP, bone morphogenetic protein; CD40, complementarity determinant 40; IL, interleukin; IFN, interferon; LIGHT, homologous to lymphotoxins, inducible expression, competes with HSV glycoprotein D for HVEM/TR2; LT, lymphotoxin; MDA-7, melanoma-differentiation associated gene-7; RANK, receptor activator of NF-κB; TGF, transforming growth factor; TNF, tumor necrosis factor; TRAIL, TNF-α related apoptosis inducing ligand (adapted from Vilcek 2003).

1.3 INTERFERON-γ

The interferon (IFN) family of cytokines is divided into Type I IFNs (IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-τ), Type II IFNs (IFN-γ) and the more recently discovered Type III IFNs (IFN-λ1-3). 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, NKT cells, B cells and dendritic cells. (Schroder *et al.* 2004, Freucht *et al.* 2001, Gessani and Bellardelli 1998). It is homodimeric, composed of two identical α-subunits, with secondary structure consisting mostly of α-helix with no β-sheet (Ealick *et al.* 1991). The biological and pathological roles of IFN-γ are discussed in more detail in the following sections. Type III IFNs are relatively uncharactised but function through a distinct receptor to that used by either Type I or II IFNs (Bartlett et al. 2005).

1.3.1 Biological functions of IFN-γ

IFN-γ has a multitude of functions, displaying direct antiviral activity through the inhibition of several stages of viral replication, as well as a variety of immunomodulatory and inflammatory roles. Mice with defective IFN-γ genes or lacking IFN-γR develop with no obvious morphological defects but display an increased susceptibility to bacterial and viral infection (van den Broek *et al.* 1995, Dalton et al 1993). Specific actions of IFN-γ signalling in inflammation and the immune system include: stimulation of antigen presentation through induced expression of Class I and II major histocompatibility complex (MHC) molecules on the surface of macrophages and T-lymphocytes; antigen processing; control of Th1/Th2 balance (primarily through inhibition of Th2 proliferation); the activation of macrophages, T-lymphocytes and NK cells 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 cells to the site of injury through increased expression of chemokines and adhesion molecules (Schroder *et al.* 2004, Tau and Rothman 1999, Stark *et al.* 1998, Boehm *et al.* 1997).

Cellular state is also influenced by IFN-γ, including regulation of 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. c-myc (Ramana *et al.* 2000)), and can either induce or suppress apoptosis depending on the cell type or state of differentiation (Schroder *et al.* 2004, Tau and Rothman 1999, Stark *et al.* 1998, Boehm *et al.* 1997). Table 1.2 summarises the biological roles of IFN-γ.

Table 1.2 Biological roles of IFN-γ

ROLE OF IFN-γ	MECHANISMS
Anti-viral activity	Inhibition of viral replication
Immunomodulation	Stimulation of antigen presentation and processing Control of Th1/Th2 balance
Inflammation	Recruitment of inflammatory cells Stimulation of cytokine production
Regulation of cellular state	Inhibition of proliferation Regulation of differentiation Regulation of apoptosis Activation of macrophages, T-lymphocytes and NK cells

1.3.2 Pathological functions of IFN-γ

The pathogenesis of several autoimmune diseases is accelerated by IFN-γ including multiple sclerosis, Type1 diabetes mellitus and HIV infection (reviewed in Billau 1996) and anti-IFN-γ has been suggested as a potential treatment for these conditions (Skurkovich and Skurkovich 2003). Multiple sclerosis is associated with a systemic increase in IFN-γ and adminstration of the cytokine to patients with the disease increases the exacerbation rate (Panitch et *al.* 1989, Panitch 1992, Navikas and Link 1996, Link *et al.* 1994). Patients with diabetes mellitus have also been shown to have elevated levels of the cytokine in blood plasma (Ozer et *al.* 2003). Non-obese diabetic (NOD) mice in which the IFN-γR gene has been knocked-out exhibit a marked inhibition of insulitis and display no signs of diabetes (Wang *et al.* 1997). Disruption of the IFN-γ gene in these mice has also been shown to delay the onset of diabetes (Serrez *et al.* 2005). Treatment of NOD mice with anti-IFN-γ antibodies, or a nonimmunogenic form of IFN-γR that similarly binds and neutralises

the cytokine, results in decreased insultis (Nicoletti *et al.* 1996, Campbell *et al.* 1991). In individuals infected with HIV, raised levels of IFN-γ have been found in the blood serum and lymph nodes and the cytokine can act as an inducer of HIV expression in monocytes (Boyle *et al.* 1993, Biswas *et al.* 1992, Griffin *et al.* 1991).

Antitumor activity has been well documented for IFN-γ. IFN-γR-null mice are highly susceptible to chemical carcinogenesis (Kaplan *et al.* 1998) and treatment with IFN-γ has been found to result in increased survival rates in animal models of tumorigenesis (Peyregne *et al.* 2004, Gansbacher *et al.* 1990). The tumor suppressive effects of IFN-γ may be due to the antiproliferative and proapoptotic effects of the cytokine and the promotion of the other immune responses (Ruiz de Almodovar *et al.* 2004, Wagner *et al.* 2004, Ikeda *et al.* 2002, Detjen *et al.* 2001, Dighe *et al.* 1994, Hawkyard *et al.* 1992). Several clinical studies have examined the use of recombinant IFN-γ as an anticancer therapy. In some cases patients exhibited tumor regression following IFN-γ treatment (Pujade-Lauraine *et al.* 1996, Boutin *et al.* 1994, Ellerhorst *et al.* 1994, Kurzrock *et al.* 1985), however, a number of trials have shown no significant effect (Elhilali *et al.* 2000, Gleave *et al.* 1998, Jett *et al.* 1994). The cytokine may also be associated with a cancer related disorder, cachexia, involving weight loss and anaemia. In this case IFN-γ treatment of mice with the condition accelerates weight loss (Ramos *et al.* 2004, Mattys *et al.* 1991).

The role of IFN-γ in the immune system and inflammation means that it is likely to have a role in disorders associated with a chronic inflammatory reaction. For instance, differential expression of IFN-γ has been linked to the pathology of glomerulonephritis (Kitching *et al.* 1999), rheumatoid arthritis (Canete *et al.* 2000) and pancreatitis (Uehara *et al.* 2003, Xie *et al.* 2001). While this report will concentrate on the implications for the development of atherosclerosis, several of the key functions contributing to disease progression may be applicable to other inflammatory conditions.

1.4 IMPORTANCE OF IFN-γ IN ATHEROSCLEROSIS

1.4.1 Atherosclerosis

Atherosclerosis is a chronic, progressive disorder characterised by the development of a complex, lipid filled plaque in the artery wall. Complications arising from atherosclerosis, including myocardial infarction and stroke, are the principal cause of death in Western societies. It is a multifactorial disease in which genetic predisposition, age, stress, physical inactivity, dietary habits, diabetes, infection, smoking, hypercholesterolemia and hypertension are some of the main risk factors (Glass and Witztum 2001, Libby 2000, Lusis 2000, Ross 1999).

The pathology of atherosclerosis can be broken down simplistically into three distinct phases: formation of fatty streaks; development of a complex lesion; and plaque rupture (Figure 1.1). In the course of the disease monocytes and T-lymphocytes migrate from the circulation into the intima of the arterial wall. The monocytes in the intima differentiate into macrophages, which then take up modified lipoproteins to transform into foam cells. Fatty streaks, often present in humans even from childhood, consist of an accumulation of such cholesterol-filled foam cells (Figure 1.1A). The more advanced, complex lesion develops as smooth muscle cells (SMCs) from the arterial media migrate into the intima. Here they may also take up lipoproteins to become foam cells (Figure 1.1B). A fibrous cap, consisting of SMCs and extracellular matrix (ECM), then forms, that encloses a necrotic core of lipid-rich debris that results from the death of accumulated foam cells (Glass and Witztum 2001, Libby 2000, Lusis 2000, Ross 1999). Other features of the complex plaque can include calcification and neovascularisation. Neovascularisation is thought to contribute to plaque instability and also provide a route by which cells can continue to enter the lesion even after the fibrous cap becomes impossible to penetrate (Lord and Bobryshev 2002).

Figure 1.2A LDL is oxidised in the subendothelial space, progressing from minimally modified LDL (mmLDL) to oxidised LDL (oxLDL). OxLDL and inflammatory cytokines induce expression of cell adhesion molecules on the endothelial surface, mediating attachment of monocytes. Monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation. Within the macrophage oxLDL cholesterol is esterified and stored in lipid droplets, converted to more soluble forms, or is exported to extracellular HDL acceptors (adapted from Glass and Witztum 2001).

Figure 1.2B Interactions between macrophage foam cells and Th1/Th2 cells establish a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and antiatherogenic effects on cells of the vessel wall. Smooth muscle cells (SMCs) migrate from the media, proliferate and secrete extracellular matrix (ECM) proteins to form a fibrous cap enclosing macrophage foam cells (adapted from Glass and Witztum 2001).

Figure 1.2C Necrosis of macrophage and SMC-derived foam cells leads to the formation of a necrotic core and accumulation of extracellular cholesterol. Macrophage secretion of matrix metalloproteinases (MMPs) and neovascularisation contribute to weakening of the fibrous plaque. Plaque rupture exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets and the formation of a thrombus (adapted from Glass and Witztum 2001).

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ACAT, acyl-CoA:cholesterol acyltransferase; apoE, apolipoprotein E; CCR2, CC chemokine receptor 2; CD36, complementarity determinant 36; CS-1, connecting segment-1; E-selectin, endothelial-selectin; HDL, high density lipoprotein; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; IFN, interferon; iNOS, inducible nitric oxide synthase; LDL, low density lipoprotein; 15-LO, 15-lipoxygenase; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage-colony stimulating factor; mmLDL, minimally modified LDL; MMP, matrix metalloproteinase; oxLDL, oxidised LDL; P-selectin, platelet-selectin; SR-A, scavenger recptor-A; Th, T helper; VCAM-1, vascular cell adhesion molecule-1.

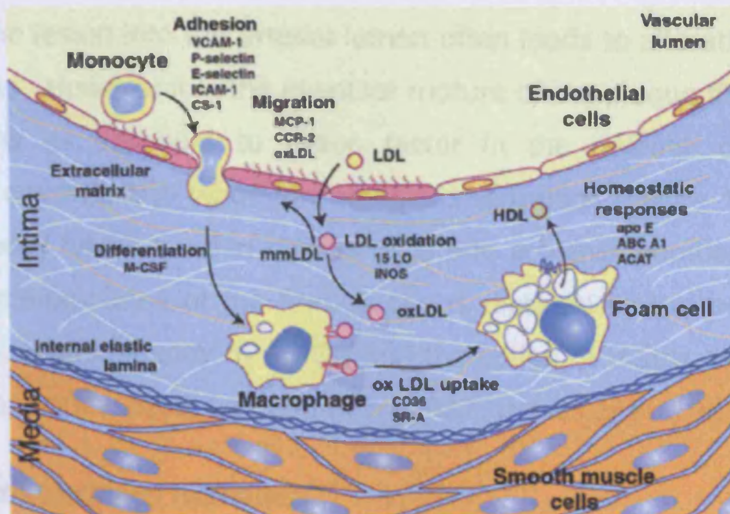


Figure 1.2A Foam cell formation

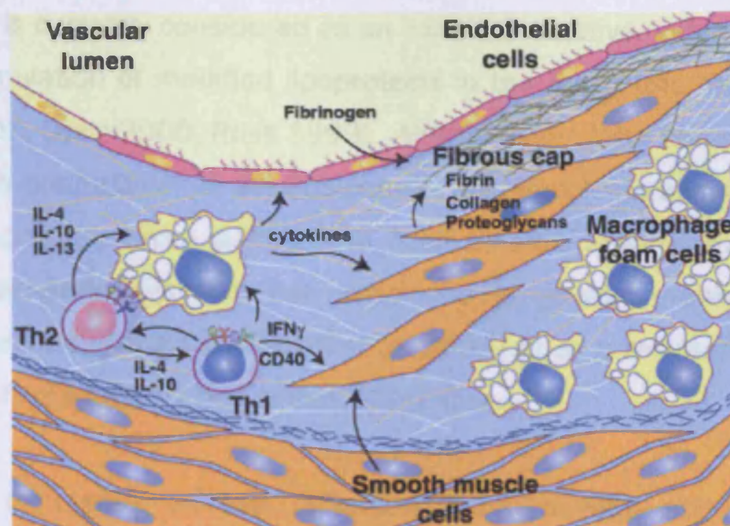


Figure 1.2B Complex plaque formation

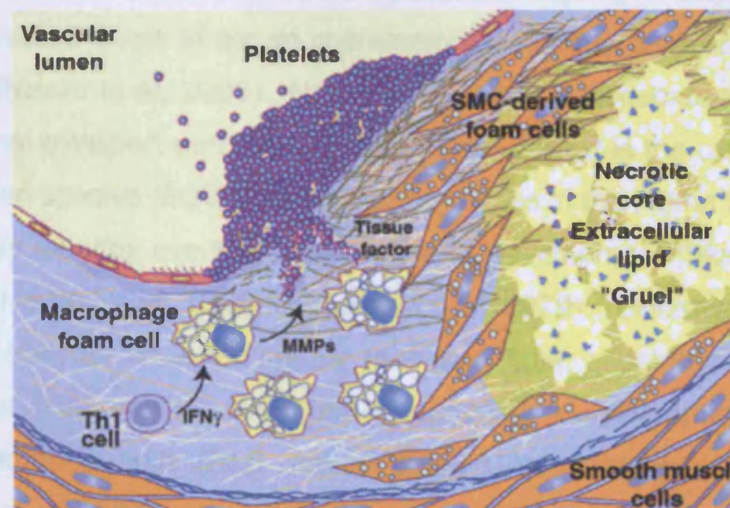


Figure 1.2C Plaque rupture and thrombosis

Growth of the lesion into the arterial lumen often leads to alterations in blood flow and ischemia. However it is the eventual rupture of the plaque that causes clinical complications as exposure to tissue factor in the necrotic core initiates the coagulation cascade and leads to thrombosis formation (Figure 1.2C). Vulnerable plaques usually have very thin fibrous caps and a higher number of inflammatory cells. The maintenance of the fibrous cap is dictated by the production of ECM components, predominantly by SMCs, and the action of matrix degrading enzymes (Lord and Bobryshev 2002, Lee and Libby 1997, Davies *et al.* 1993).

1.4.2 Response to injury hypothesis

The "response to injury" hypothesis is a widely accepted model for atherogenesis. The disease is currently considered as an initially protective, inflammatory response to the accumulation of modified lipoproteins in the artery (the "injury") (Glass and Witztum 2001, Lusis 2000, Ross 1999). Although many risk factors are believed to influence the progression of atherosclerosis, hypercholesterolemia is undoubtedly the most important, being sufficient in itself to drive lesion formation (Steinberg 2002). Hypercholesterolemia can arise due to genetic predisposition (*e.g.* in familial hypercholesterolemia) (Lusis *et al.* 2004) or as a result of various lifestyle factors including a high fat diet or lack of exercise.

Cholesterol is carried in the bloodstream by several lipoprotein particles: chylomicrons; very low-density lipoprotein (VLDL); low-density lipoprotein (LDL); and high-density lipoprotein (HDL). LDL transports the majority of serum cholesterol in humans. Elevated levels of serum cholesterol lead to the deposition of LDL in the arterial wall (Navab *et al.* 1996). Native LDL would not accumulate rapidly enough through normal transport pathways to form foam cells. It is the oxidation of LDL by reactive oxygen species (ROS) and oxidative enzymes in the intima that is thought to be the primary intiating event in atherosclerosis development (Skalen 2002, Cyrus *et al.* 1999, Navab *et al.* 1996, Goldstein *et al.* 1979). Oxidatively modified LDL (oxLDL) acts both as an inflammatory mediator itself; capable of stimulating the recruitment of inflammatory cells, and by inducing an inflammatory response in overlying endothelial cells (ECs) (Navab *et al.* 1996, McMurray *et al.* 1993, Yui 1993, Quinn *et al.* 1987).

1.4.3 Role of endothelial cells in atherosclerosis

In response to oxLDL and cytokines within the intima, the endothelium becomes more permeable and displays increased levels of cell adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) (Cybulsky and Gimbrone 1991), intercellular adhesion molecule-1 (ICAM-1) and P- and E-selectins (Collins *et al.* 2000, Dong *et al.* 1998). The endothelium also produces a range of vasoactive molecules, cytokines, chemokines and growth factors. These include IL-1, IL-18, TNF-α, IL-8, MCP-1, macrophage-colony stimulating factor (MCSF), PDGF and TGF-β. This response leads to increased leukocyte adhesion to the endothelium and the extravasation of monocytes and T-lymphocytes into the intima, contributing to lesion development. The further release of cytokines, chemokines and growth factors by these cells enhances the endothelial response. Certain cytokines (*e.g.* IL-3, IL-18) and chemokines (*e.g.* IL-8, MCP-1) stimulate the proliferation of ECs and promote angiogenesis (Raines and Ferri 2005, Grainger 2004a, Fazio and Linton 2001, Ross 1999).

An important regulator of EC gene expression is shear stress, controlling transcription through shear stress regulatory elements (SSREs) (consensus sequence: GAGACC) in the promoter regions of target genes. The endothelium appears to be sensitive to different flow forms in the artery which cause varying patterns in gene expression. Steady laminar flow is characterised as atheroprotective, and disturbed flow as an atheroprone waveform. This is important in explaining the prevalence of atherogenesis at particular arterial sites, such as branches or curvatures where the blood flow is altered. These sites display an increased accumulation of T-cells and NKT cells; high concentrations of inflammatory cytokines; and increased permeability of the endothelium to lipoprotein molecules. Plaques forming in these regions often have a high cellularity and are predisposed to the generation of thin fibrous caps prone to rupture. Gene array analysis has shown that atheroprone waveforms upregulate the expression of cell surface adhesion molecules (*e.g.* VCAM-1, ICAM-1, E-selectin), chemokines and chemokine receptors (*e.g.* MCP-1, IL-8, CXCR4), pro- and antioxidant enzymes (lipoxygenases, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD), nitric oxide synthase (NOS)), and matrix degrading enzymes (*e.g.* MMP-1) in ECs (Dai *et al.* 2004, Vanderlaan *et al.* 2004, Brooks *et al.* 2002, Chen *et al.* 2001, Gimbrone 1999).

1.4.4 Role of monocytes and macrophages in atherosclerosis

Monocytes and macrophages are present at all stages of lesion development and make up about 60-70% of cells in the advanced plaque. They have a multifunctional role in the progression of the lesion (reviewed in Takahashi *et al.* 2002). Monocytes recruited to the site of inflammation infiltrate the artery wall where they are propagated and differentiate into macrophages in response to stimulation by M-CSF (Watanabe *et al.* 1995). OxLDL has been shown by microarray analysis, to induce the expression of a number of chemokines and other cytokines in macrophages that contribute to inflammation within the lesion and the further recruitment of immune cells (Mikita *et al.* 2001). Initially the recruitment of macrophages acts as a protective mechanism through the uptake and therefore removal of the injurious agent, oxLDL. However macrophage uptake of modified lipoproteins ultimately leads to foam cell formation and atherogenesis. Phagocytosis of cholesterol-loaded apoptotic cells by macrophages leads to further accumulation of lipid within these cells (Li and Glass 2002, Kruth 2001). Mice carrying a mutation in the M-CSF gene (the osteopetrotic mutation) have very low circulating numbers of macrophages. When crossed with apoE-deficient mice (that rapidly develop atherosclerotic lesions on a high cholesterol diet) the offspring show a high resistance to the development of atherosclerotic plaques, supporting a proatherogenic view of macrophages (Smith *et al.* 1995).

1.4.4.1 Macrophage foam cell formation

The uptake of oxLDL to form foam cells is mediated predominantly through scavenger receptors on the macrophage surface. The most important of these in atherosclerosis are scavenger receptors A (SR-A) and CD36 (Yamada *et al.* 1998, Suzuki *et al.* 1997), both of which are upregulated by exposure to oxLDL (Nakgawa *et al.* 1998, Yoshida *et al.* 1997). The LDL receptor (LDL-R) also initially contributes to lipid loading by binding to minimally modified LDL (mmLDL). However, LDL-R is downregulated by the sterol regulatory element-binding protein (SREBP) pathway as cholesterol accumulates, while scavenger receptors continue to take up increasing amounts of lipoprotein (Goldstein *et al.* 2002, Brown and Goldstein 1999). The VLDL receptor (VLDL-R) and LDL-R-like protein (LRP) are also upregulated in

atherosclerotic lesions and may contribute to the pathogenesis of the disease as they have a high affinity for atherogenic remnant particles and are involved in the uptake of modified LDL (Schulz *et al.* 2003, Kosaka *et al.* 2001, Hiltunen *et al.* 1998).

As macrophage levels of cholesterol increase, efflux mechanisms may maintain homeostasis for a short while largely through a process known as reverse cholesterol transport. One of the principal components of this system is the ATP-binding cassette transporter A1 (ABCA1), that mediates transfer of cholesterol from the cell to HDL, a role that makes HDL highly atheroprotective (reviewed in Tall *et al.* 2002). ApoE-null mice transplated with ABCA1-deficient bone marrow cells showed significantly more atherosclerosis than those given wild type bone marrow (Aiello *et al.* 2003, Aiello *et al.* 2002). Ultimately however, in high concentrations of modified LDL, the macrophage fails to maintain an adequate cholesterol balance. The majority of cholesterol in foam cells exists in the form of cholesterol esters. In the absence of a suitable acceptor (such a HDL), the enzyme acyl coenzyme A: acylcholesterol transferase (ACAT) catalyses the formation of cholesterol esters from free intracellular cholesterol and long chain fatty acids. Raised levels of ACAT1, the isoform principally found in macrophages, are associated with these cells in the atherosclerotic plaque (Miyazaki *et al.* 1998). It was originally thought that knocking out the ACAT1 gene would be beneficial to atherosclerosis but apoE-null mice with this deletion exhibited atherosclerosis progression to a similar extent to those without it. Though the lesions had a reduced cholesterol content, deposits were found elsewhere including the brain (Fazio *et al.* 2001). Partial inhibition of ACAT activity however has been demonstrated to have an atheroprotective effect in apoE-null mice (Kusunoki *et al.* 2001).

1.4.4.2 Plaque destabilisation

Lendon *et al.* (1991) showed that lesions with a high macrophage density had weaker fibrous caps than less cellular lesions. Macrophages may contribute to the destabilisation of the plaque in a number of ways. As macrophage foam cells undergo apoptosis themselves, their contents contribute to the necrotic core (Li and Glass 2002). A correlation has been found between lesional macrophages expressing the marker of apoptosis, caspase 3, and high levels of expression of

tissue factor. The release of prothrombotic molecules, such as tissue factor and complement proteins (*e.g.* complement C3b) produced by macrophages, into the necrotic core following foam cell apoptosis increases the thrombogenic potential of the plaque (Hutter *et al.* 2004, Takahashi *et al.* 2002). Macrophages also produce proteolytic enzymes, including matrix metalloproteinases (*e.g.* MMP-2, MMP-9) that act to weaken the fibrous cap and so lead to eventual plaque rupture (Galis *et al.* 1994).

1.4.5 Cytokines in atherosclerosis

The inflammatory component of atherosclerosis has come to prominence in recent years and cytokines have emerged as key regulators in the development of the atherosclerotic lesion. Cell types within the atheroma include monocytes/macrophages, ECs, SMCs, and T-lymphocytes as well as lower numbers of dendritic cells (DCs) and in some cases B-cells. A variety of cytokines and chemokines are secreted by each of these cell types within and around the site of plaque formation and they are involved in all stages of atherogenesis. Cytokines affect the recruitment of cells to the lesion through: chemotaxis; modulation of the expresson of cell adhesion molecules by ECs and SMCs; and the regulation of cell proliferation and migration in the atheroma. Cholesterol loading of foam cells is modulated by the cytokine-induced regulation of genes involved in the uptake of oxLDL. Cytokines also contribute to the overall stability of the lesion by regulating the expression of effectors of ECM turnover, and to thrombosis through the regulation of genes involved in coagulation cascades. Recent reviews discuss in detail the role of cytokines in the pathogenesis of atherosclerosis and other cardiovascular diseases (Mehra *et al.* 2005, Grainger 2004a, Young *et al.* 2002). In atherosclerosis the impact of proinflammatory stimuli (*e.g.* TNF-α, IL-12 and IL-18), that are predominantly proatherogenic (Branen *et al.* 2004, Elhage *et al.* 2003, Bruunsgaard *et al.* 2000, Lee *et al.* 1999), appears to outweigh that of anti-inflammatory, antiatherogenic cytokines such as TGF-β and IL-10 (Grainger 2004b, Zimmerman *et al.* 2004, Mallat *et al.* 1999) triggering a chronic inflammatory response (Frostegard *et al.* 1999). However, the wide range of effects induced by each of these mediators means that a single molecule may have both pro- and anti-atherogenic roles (*e.g.* IFN-γ (Section 1.4.6)).

1.4.6 Effects of IFN-γ on atherogenesis

Among the cytokines, IFN-γ is emerging as a key factor in the pathogenesis of atherosclerosis. Immunohistochemical studies have revealed the localisation of IFN-γ to the atherosclerotic lesion where it is secreted primarily by activated T-lymphocytes (CD4+ Th1 cells) and possibly macrophages (Gessani and Bellardelli 1998, Hansson *et al.* 1989). Plaque formation is most likely to occur at vascular branch ponts where blood flow is altered. Th1 cells, believed to be largely proatherogenic, accumulate in these regions and the expression of IFN-γ is induced (Dai *et al.* 2004, Daugherty and Rateri 2002, Gimbrone 1999, van der Wal *et al.* 1994). More recently a key role for NKT cells, which exhibit the properties of both NK cells and T cells and secrete high levels of IFN-γ, has been identified in atherosclerosis. Mice with low numbers of NKT cells display less atherosclerosis development than the wild type counterparts (Aslanian *et al.* 2005, Nakai *et al.* 2004). IFN-γ has a range of influences on disease progression, acting on all the major cell types of the plaque and the overall effect is complex. A large number of genes are directly or indirectly regulated by IFN-γ including approximately 25% of the macrophage transcriptome (Ehrt *et al.* 2001). The expression of many such genes impacts on several areas of lesion development. While many of the changes in gene expression are proatherogenic there are also a number of key genes for which the regulatory effects of IFN-γ are antiatherogenic.

1.4.6.1 Proatherogenic roles of IFN-γ

1.4.6.1.1 Recruitment of immune cells to the lesion

Growth of the atheroma occurs largely through continued recruitment of macrophages and T-lymphocytes, and also SMC migration and proliferation. The lesions of apoE- or LDL-R-null mice deficient in IFN-γ or its receptor have a dramatically reduced cellular content, indicating an important role for IFN-γ in this recruitment (Buono *et al.* 2003, Gupta *et al.* 1997). The secretion of chemokines by lesional cells has an important role in the development of atherosclerosis, discussed in Section 1.5. IFN-γ is responsible for the induction of a number of key

chemokines and chemokine receptors including: MCP-1, MIP-1α and -β (Valente *et al.* 2001, Martin and Dorf 1991) and the receptors CCR5, CCR1 and CCR3 (Hariharan *et al.* 1999, Zella *et al.* 1998); the CXC chemokines IP-10, Mig, I-TAC and CXCL16 (Wuttge et al. 2004, Cole *et al.* 1998, Farber 1997); and the CX3C chemokine fractalkine (Imaizumi *et al.* 2000).

The uptake of cells into the blood vessel intima at the site of the plaque also requires cell adhesion molecules displayed on the luminal surface of the endothelium. One of the earliest events in the development of the fatty streak is the upregulation of members of the immunoglobulin family of adhesion molecules including VCAM-1 and ICAM-1 (Collins *et al.* 2000, Cybulsky and Gimbrone 1991). IFN-γ is an important mediator of this response, inducing the expression of both VCAM-1 and ICAM-1 in ECs and SMCs (Chung *et al.* 2002, Cybulsky *et al.* 1993). IFN-γ also increases the expression of integrins such as α-5-β-1 integrin, on the surface of VSMCs. This allows the cells to bind to the fibronectin component of the extracellular matrix (ECM) and hence provides a substrate for SMC migration to the lesion and causes the cells to adopt a proliferative phenotype (Barillari *et al.* 2001).

The majority of macrophages and T-cells in the atherosclerotic lesion are in an activated state whereby they are primed to respond to stimuli such as bacterial antigens (or in the lesion, oxLDL). For both cell types this involves the increased secretion of proinflammatory cytokines and chemokines so contributing to the inflammation and growth of the plaque. IFN-γ is known to stimulate both the differentiation of monocytes to macrophages and macrophage activation (Nathan *et al.* 1983). Activation of CD4+ T-cells by IFN-γ occurs through increased antigen presentation by MHC Class II molecules. While both Type I and Type II IFNs cause upregulation of MHC Class I antigens that present antigens for the activation of CD8+ T-cells, IFN-γ alone induces MHC Class II expression involved in the activation of CD4+ T-cells (Th1). IFN-γ increases the expression of MHC Class II molecules on the surface of a variety of cell types including ECs, macrophages and SMCs (Iwata *et al.* 2001, Mach *et al.* 1996, Jonasson *et al.* 1985).

1.4.6.1.2 Cholesterol accumulation in foam cells

The uptake of cholesterol, in the form of oxLDL, by macrophages and SMCs to form lipid-loaded foam cells, is a key process throughout all the stages of plaque development. IFN-γ has a regulatory effect on the expression of several genes that are key players in cholesterol metabolism, including the downregulation of ABCA1, apolipoprotein E and cholesterol hydroxylase and upregulation of ACAT (Reiss *et al.* 2001, Panousis and Zuckerman 2000a, Panousis and Zuckerman 2000b, Brand *et al.* 1993) (Section 1.4.4.1). It appears that a general effect of IFN-γ may be to increase the lipid loading of foam cells via a downregulation of cholesterol efflux pathways and up-regulation of mechanisms by which cholesterol accumulates (*e.g.* esterification through ACAT). Incubation of foam cells with IFN-γ decreases HDL-mediated cholesterol efflux by approximately 2 fold and efflux to apoA1 by more than 4 fold (independent of apoE or SR-B1 synthesis). Overall, treatment has been shown to result in a redistribution of intracellular cholesterol and an increase in cholesterol ester accumulation (Panousis and Zuckerman 2000a, Panousis and Zuckerman 2000b). More recently, IFN-γ has been shown to impede reverse cholesterol transport and promote foam cell transformation in human THP-1 monocytes/macrophages (Reiss *et al.* 2004).

14.6.1.3 Complex plaque formation and thrombosis

As the disease progresses, the atherosclerotic lesion becomes increasingly complex and the advanced plaque may include features such as calcification and neovascularisation (Lord and Bobryshev 2002). Neovascularisation and angiogenesis may be increased by IFN-γ by the upregulation of certain chemokines (*e.g.* MCP-1). The cytokine may have a role in mechanisms of calcification through an increase in the expression of 1-α-hydroxylase. This enzyme is responsible for catalysing the conversion of 25-hydroxyvitamin D to 1-α, 25-dihydroxyvitaminD, an active metabolite of vitamin D that contributes to calcification (Esteban *et al.* 2004, Shoi *et al.* 2000).

Production of ECM components by SMCs contributes to the formation of a fibrous cap on the luminal side of the lesion. Beneath this cap, the advanced plaque contains a lipid filled necrotic core, formed as foam cells in the lesion die and

release their contents. Cell death in the atherosclerotic lesion occurs primarily through the process of apoptosis. The function of IFN-γ in apoptosis is complex and the cytokine can have either a pro- or antiapoptotic effect depending on the specific state of the cell (Schroder *et al.* 2004, Tau and Rothman 1999, Stark *et al.* 1998, Boehm *et al.* 1997). Inagaki *et al.* (2002) have shown that IFN-γ stimulates the apoptosis of macrophage foam cells. Microarray studies have revealed a number of IFN-stimulated genes (ISGs) with apoptotic functions including TNF-α related apoptosis inducing ligand (TRAIL), Fas and caspases 4 and 8 in macrophages. Upregulation of caspases 1 and 3 by IFN-γ has also been demonstrated in macrophages. In SMCs and monocytes an increase in susceptibility to apotosis and IL-1β-converting enzyme (ICE) expression has been observed in response to IFN-γ treatment (Inagaki *et al.* 2002, Tamura *et al.* 1996, Chawla-Sarkar *et al.* 2003).

Certain factors contribute to the likelihood of plaque rupture, leading to the clinical complications of atherosclerosis, by affecting the composition and stability of the fibrous cap. IFN-γ producing Th1 cells frequently accumulate at the sites of plaque rupture and there are several mechanisms by which IFN-γ can contribute to destabilisation of the plaque (van der Wal *et al.* 1994). The fibrous cap is composed principally of ECM components synthesised by SMCs, including elastic filament and proteoglycans with collagens providing most of the tensile strength. Lesions with a high foam cell content and low ECM have been shown to be more likely to rupture and cause thrombosis formation (Lee and Libby 1997, Davies *et al.* 1993). Increased cellularity of the lesion caused by exposure to IFN-γ has already been discussed. Additionally the expression of a number of collagen genes (*e.g.* collagens 1 and 3), as well as SMC proliferation and matrix synthesis, is inhibited by IFN-γ (Yuan *et al.* 1999, Amento *et al.* 1991, Hansson *et al.* 1988).

Matrix metalloproteinases (*e.g.* MMP-1, 2, 3 and 9) break down the ECM and thus cause plaque destabilisation (Galis *et al.* 1994). Activated Th1 cells have been shown to stimulate increased production of these MMPs by macrophages and SMCs (Schonbeck *et al.* 1997). Tissue factor activity is also enhanced by IFN-γ treatment, in synergy with C-reactive protein (CRP), so increasing the likelihood or rate of thrombosis following plaque rupture (Nakagomi *et al.* 2000).

1.4.6.2 Antiatherogenic roles of IFN-γ

Despite the range of proatherogenic functions of IFN-γ discussed so far, certain modulations in gene expression caused by this cytokine appear to be atheroprotective. Treatment of monocyte/macrophage cells with IFN-γ *in vitro* has been demonstrated, in some cases, to reduce both the oxidation of LDL and the uptake of oxLDL (Folcik *et al.* 1997, Christen *et al.* 1994, Fong *et al.* 1994, Fong *et al.* 1990). A recent study has also found that transplantation of IFN-γ-deficient bone marrow in LDL-R-/- mice increases the extent of atherosclerosis development, suggesting that IFN-γ secreted by bone marrow derived cells (T-lymphocytes and monocytes/macrophages) has an atheroprotective effect. Notably however the lesions in these mice had a high collagen content suggesting that they were less susceptible to rupture (Niwa *et al.* 2004). The seemingly contradictory pro- and antiatherogenic roles of IFN-γ can be compared to the cytokine's similarly conflicting pro- and antiinflammatory effects. While IFN-γ is primarily considered to be a proinflammatory cytokine, it induces the expression of a number of anti-inflammatory mediators (*e.g.* IL-1 receptor antagonist and IL-18 binding protein) in a range of cell types (Muhl and Pfeilschifter 2003).

1.4.6.2.1 Reduced uptake of oxLDL

In atherosclerosis, scavenger receptors, most significantly SR-A and CD36, are the principal mediators of oxLDL uptake in foam cell formation (Yamada *et al.* 1998, Greaves *et al.* 1998, Suzuki *et al.* 1997). IFN-γ mediates inhibition of the expression of SR-A and CD36 in macrophages (although it increases expression in SMCs) and it is primarily to this effect that reductions in macrophage foam cell formation are attributed (Nakagawa *et al.* 1998, Geng and Hansson 1992). VLDL-R and LRP have also been implicated in atherosclerosis development through the uptake of modified LDL and atherogenic remnant particles (Schulz *et al.* 2003, Hiltunen *et al.* 1998). The expression of these receptors is similarly repressed by IFN-γ (Kosaka *et al.* 2001, LaMarre *et al.* 1993).

The physiological role of LPL in lipid metabolism is to catalyse the hydrolysis of the triacyl glycerol component of VLDL and chylomicrons to provide non-esterified fatty acids and 2-monoacylglycerol for tissue utilisation (Mead *et al.* 2002). LPL plays a compelx role in atherogenesis with both pro- and antiatherogenic actions being reported. The enzyme expressed by adipose tissue and muscle is considered to be antiatherogenic because it aids the clearance of circulating lipoproteins. On the other hand, LPL expressed by macrophages is proatherogenic. The main evidence for this comes from a series of bone marrow transplantation studies (reviewed in Stein and Stein 2003, Mead and Ramji 2002, Mead *et al.* 1999). For example, mice transplanted with LPL-null bone marrow (therefore producing LPL-deficient macrophages) exhibit decreased macrophage foam cell development in atherosclerosis (Babaev *et al.* 1999). It is believed that the lipolysis of VLDL and chylomicrons by LPL increases the formation of atherogenic lipoprotein remnants. Additionally, the enzyme on the surface of macrophages acts as a molecular bridge, assisting the uptake of oxLDL to form foam cells (Stein and Stein 2003, Mead and Ramji 2002, Mead *et al.* 1999, Zilversmit 1973). We have shown previously that IFN-γ reduces LPL gene expression in macrophages at the transcriptional level, and this represents an atheroprotective effect (Hughes *et al.* 2002, Tengku-Muhammed *et al.* 1999a, Tengku-Muhammad 1996).

1.4.6.2.2 Effects on oxidative stress in the vascular wall

In certain studies, IFN-γ has been found to inhibit macrophage-mediated LDL oxidation, a crucial step in the development of atherosclerosis (Folcik *et al.* 1997, Christen *et al.* 1994, Fong *et al.* 1994). IFN-γ has various effects that may contribute to changes in oxidative stress in the vascular wall and thus affect the oxidation of LDL (Madamanchi 2005). Of particular importance is the regulation of nitric oxide (NO) production. Signalling by IFN-γ, in particular in synergy with lipopolysaccharide (LPS) or other cytokines (*e.g.* TNF-α, IL-2, TGF-β, IL-4), leads to an upregulation of inducible NOS (iNOS) gene expression in a wide variety of cell types, including ECs, SMCs and macrophages (Yokoyama *et al.* 1996, Hukkanen *et al.* 1995). The subsequent increase in NO plays a role in mediating many of the antiviral and antimicrobial effects of IFN-γ, participating directly in the killing of parasitic cells (Bogdan 2001).

antiviral and antimicrobial effects of IFN-γ, participating directly in the killing of parasitic cells (Bogdan 2001).

Increased generation of NO, which acts as a potent antioxidant, in response to IFN-γ may be responsible for observations that the cytokine counteracts the oxidation of LDL (Jessup and Dean 1993). Indeed, iNOS-deficient mouse macrophages disclose a prooxidant effect of IFN-γ on LDL oxidation (Niu *et al.* 2000). NO also has a number of other antiatherogenic effects, including: inhibition of immune cell recruitment to the lesion by counteracting the increase in VCAM-1 expression on ECs; blocking nuclear factor-kappa B (NF-κB) signalling which is important for many proinflammatory pathways; and decreasing the proliferation of VSMCs and both T-cell activation and proliferation, leading to reduced cytokine production (including IFN-γ) (Gewaltig and Kojda 2002, Bodgan 2001, De Catarina *et al.* 1995, Peng *et al.* 1995). Knock-out of endothelial NOS (eNOS) in mice has been shown to increase atherosclerotic lesion formation (Knowles *et al.* 2000). It should, however, be noted that at high vascular concentrations of NO (appr. >1μM), a reaction is promoted with superoxide to form peroxynitrite, an oxidant stronger than superoxide itself (Beckman and Koppenol 1996). In fact, where eNOS is overexpressed it has been shown to accelerate atherosclerotic lesion formation in apoE-deficient mice (Kawashima and Yokoyama 2004, Ozaki *et al.* 2002, O'Donnell and Freeman 2001). It has also been suggested that graft arteriosclerosis is promoted by IFN-γ through the induction of iNOS expression leading to excessive production of NO (Mitchell and Lichtman 2004).

IFN-γ also regulates the expression of a number of other genes implicated in the control of oxidative stress and the oxidation of LDL. A consensus on the action of such gene regulation has, however, not emerged as some effects are atheroprotective whereas others are proatherogenic. For example, IFN-γ induces the expression of extracellular (EC)-SOD, which protects cells from oxidative stress via the removal of reactive oxygen species (ROS) (Stralin and Marklund 2000, Takahashi *et al.* 1998, Marklund 1992). In addition, the expression of myeloperoxidase, implicated in the development of atherosclerosis through the production of ROS, is suppressed by IFN-γ (Zhang *et al.* 2001, Podrez *et al.* 2000,

Kawano *et al.* 1993). Furthermore, IFN-γ decreases the expression of 15-lipoxygenase, which has been linked to the oxidation of LDL (Li *et al.* 2005, Takahashi *et al.* 2005b, Fong *et al.* 1994, Conrad *et al.* 1992). On the other hand, the cytokine has been demonstrated to stimulate the secretion of ROS by macrophages, ECs and neutrophils (Tennenberg *et al.* 1993, Matsubara and Ziff 1986, Murray *et al.* 1985). This may be because of the induced expression of vascular enzymes such as NADPH oxidase (Lopez *et al.* 2003, Obermeier *et al.* 1995, Pfefferkorn *et al.* 1990) and xanthine oxidase (Ghezzi *et al.* 1984).

1.4.6.3 Mouse models

Compound-deficient mice in apoE and IFN-γ or IFN-γR exhibit significant reductions in diet-induced atherosclerosis compared with apoE-null mice (Whitman *et al.* 2002a, Gupta *et al.* 1997). Recently, compound-deficient mice in LDL-R and IFN-γ have also been shown to exhibit decreased atherosclerosis compared to LDL-R-null mice (Buono *et al.* 2003). Furthermore, administration of exogenous IFN-γ enhances atherosclerosis in apoE-deficient mice and IL-18 has also been shown to enhance atherosclerosis in these mice through the release of IFN-γ (Whitman *et al.* 2002b, Whitman *et al.* 2000). Interestingly, IFN-γ has been found to elicit arteriosclerosis in the absence of leukocytes, suggesting that a proatherogenic effect may be mediated through actions on VSMCs (Tellides *et al.* 2000). As mentioned previously, Th1 and NKT cells, both of which secrete large amounts of IFN-γ and localise to sites of lesion formation, have been shown through mouse models to be proatherogenic (Aslanian *et al.* 2005, Nakai *et al.* 2004, Daugherty and Rateri 2002). Inhibition of Th1 polarization leading to decreased IFN-γ secretion is also atheroprotective (Laurat *et al.* 2001). In a directly clinically relevant situation, there is a reduced incidence and severity of transplant arteriosclerosis following heart grafts in IFN-γ-deficient mice (Raisanen-Sokolowski *et al.* 1998, Nagano *et al.* 1997)

Table 1.3 Pro- and antiatherogenic effects of IFN-γ

PROATHEROGENIC				ANTIATHEROGENIC			
GENE REGULATION (+/-)	CELL TYPE	EFFECT	REFERENCES	GENE REGULATION (+/-)	CELL TYPE	EFFECT	REFERENCES
Cell adhesion molecules (+) (e.g.VCAM-1,ICAM-1)	EC SMC	Recruitment inflammatory cells	Cybulsky *et al.* 1993 Chung *et al.* 2002	SRs (-) (e.g. SR-A, CD36)	Macrophage	Reduced uptake oxLDL	Geng and Hansson 1992
Chemokines (+) (e.g. MCP-1, MIP-1α, MIP-1β)	Macrophage EC	Recruitment inflammatory cells	Valente *et al.* 1998 Martin and Dorf 1991	LRP (-)	Macrophage	Reduced uptake oxLDL	Kosaka *et al.* 2001
MHC-II (+)	Macrophage EC SMC	Antigen presentation T cell activation	Iwata *et al.* 2001 Jonasson *et al.* 1985 Mach *et al.* 1996	VLDLR (-)	Macrophage	Reduced uptake oxLDL	LaMarre *et al.* 1993
ABCA1 (-)	Macrophage	Reduced cholesterol efflux	Panousis and Zuckerman 2000a	LPL (-)	Macrophage	Reduced uptake oxLDL Reduced production of proatherogenic remnants	Hughes *et al.* 2002
ApoE (-)	Macrophage	Reduced cholesterol efflux	Brand *et al.* 1993	iNOS (+)	Macrophage EC SMC	Reduced oxidation LDL	Yokoyama *et al.* 1996
ACAT (+)	Macrophage	Increased cholesterol esterification	Panousis and Zuckerman 2000b				
Cholesterol hydroxylase (-)	Macrophage EC	Reduced cholesterol metabolism	Reiss *et al.* 2001				
MMPs (+) (e.g. 1, 2, 3, 9)	Macrophage SMC	Plaque instability	Schonbeck *et al.* 1997				
Collagens (-) (e.g.-1, -3)	Macrophage SMC	Plaque instability	Amento et al 1991 Yuan *et al.* 1999				

Abbreviations: ABCA1, ATP-binding cassette transporter-A1; apoE, apolipoprotein E; CD36, complementarity determinant 36; EC, endothelial cell; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; LPL, lipoprotein lipase; LRP, low density lipoprotein-receptor related protein; MCP-1, monocyte chemoattractant protein-1; MHC-II, major histocompatibility antigen class-II; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; oxLDL, oxidised-LDL; SMC, smooth muscle cell; SR-A, scavenger recptor-A; VCAM-1, vascular cell adhesion molecule-1; VLDL-R, very low density-lipoprotein-receptor

In contrast to these findings, a recent study by Niwa *et al.* (2004) suggested that IFN-γ produced by bone marrow-derived cells delayed the progression of atherosclerosis. The results in relation to changes in lipoprotein levels have also been controversial. For example, exogenous administration of IFN-γ in apoE-deficient mice decreases serum cholesterol levels but enhances atherosclerosis formation (Whitman *et al.* 2000). Another study observed a gender specific effect whereby IFN-γ-deficiency in male, but not female, apoE-null mice was associated with reduced atherosclerosis development (Whitman *et al.* 2002). Table 1.4 summarises the findings from studies into the effect of IFN-γ on atherosclerotic lesion formation in mice.

The role of IFN-γ in the pathology of atherosclerosis is undeniably complex and the proatherogenic versus antiatherogenic nature of the cytokine has long been a subject for debate. While it is still not currently possible to reconcile all the conflicting evidence, the majority of research from mouse models of the disease that are deficient in IFN-γ signalling points towards a largely proatherogenic role. However, such a plethora of genes are regulated by IFN-γ that it is hard to determine the impact of each of these on the disease. Substantial evidence exists that some of these gene regulatory events are antiatherogenic. It is possible that the role of IFN-γ may depend on the stage of the pathology and the presence of other factors in the atheroma, and mice that are deficient in IFN-γ signalling represent an extreme situation. Further research is, therefore, clearly needed into the phenotypic effects of this cytokine on various areas of complex plaque growth (e.g. foam cell formation, angiogenesis, calcification, plaque rupture) and into the specific signalling pathways involved in the regulation of individual genes, with the hope of identifying potential therapeutic targets in order to combat this disease.

Table 1.4 Effects of IFN-γ on atherosclerotic lesion formation in mice

REFERENCE	GENE KNOCK-OUT	TREATMENTS/ PROCEDURES			EFFECT OF ABSENCE OF IFN-γ SIGNALLING/ ADMINISTRATION OF IFN-γ ON ATHEROGENESIS	CONCLUSIONS: ROLE OF IFN-γ IN ATHEROSCLEROSIS
		Cytokine treatment	*Transplant*	*Diet*	*Changes*	
Gupta *et al.* 1997	IFN-γR/ ApoE			High fat	Decreased lesional area Decreased lipid content Decreased cellularity Increased collagen content Increased apoAIV rich particles in plasma	Proatherogenic
Nagano *et al.* 1997	IFN-γ		Heart transplant in immuno-suppressed mice		Decreased intimal thickening	Promotes graft arteriosclerosis
Raisanen-Sokolowski *et al.* 1998	IFN-γ		Heart transplant in immuno-suppressed mice		Decreased intimal thickening Increased cellularity	Promotes graft arteriosclerosis but may increase plaque stability in atherosclerosis
Tellides *et al.* 2000		IFN-γ injection (3 times weekly)	Transplant pig/human arteries in immuno-deficient mice		Thickening of intima Accumulation VSMCs in intima	Can induce arteriosclerotic changes through action on VSMCs
Whitman *et al.* 2000	ApoE	IFN-γ injection (daily)			Increased lesional area Increased cellularity Decreased collagen content Decreased serum cholesterol	Proatherogenic but may decrease serum cholesterol levels
Whitman *et al.* 2002	IFN-γ/ ApoE	IL-18 injection (daily)			IL-18 induced atherogenesis not observed in compound knock-out mice	Proatherogenic
Whitman *et al.* 2002	IFN-γ/ ApoE			Normal/ high fat	Decreased lesional area in males only Decreased cellularity in males only	Proatherogenic in male mice
Buono *et al.* 2003	IFN-γ/ LDLR			High fat	Decreased lesional area Decreased lipid content Decreased cellularity at 8 weeks	Proatherogenic
Niwa *et al.* 2004	IFN-γ (BMT)/ LDLR		BMT	High fat	Increased lesional area at 6 weeks Decreased cellularity Increased collagen content	IFN-γ produced by BM-derived cells anti-atherogenic but may decrease plaque stability

Abbreviations: BMT, bone marrow transplant; IFN-γ, interferon-γ; IL-18, interleukin-18; LDL-R, low density-lipoprotein-receptor; VSMC, vascular smooth muscle cell.

1.5 CHEMOKINES

As discussed previously, chemokines have an important role in the progression of atherosclerosis. IFN-γ alters the expression of a number of chemokines and chemokine receptors and these responses are likely to impact on the development of the disease.

1.5.1 Structure and classification

Chemokines are a class of cytokines best characterised with respect to their role in the recruitment of immune cells to the site of inflammation. They are small (most 8-10kDa) disulphide linked polypeptides classified according to the arrangement of a conserved N-terminal cysteine motif. Disulphide bonds link the first and third and the second and fourth cysteine residues in the motif. The largest chemokine family is the CC chemokines in which the first two cysteine residues are adjacent; in CXC chemokines they are separated by one amino acid; and in CX3C chemokines, of which there is only one known at present (fractalkine), they are separated by three amino acids. The C chemokines, of which two have been identified at present (lymphotactin and SCM-1β), have only one pair of cysteine residues linked by a disulphide bond. The majority of chemokines function as dimers although one, platelet factor (PF) 4 (CXCL4) is a tetramer (IUIS/WHO Subcommitte on Chemokine Nomenclature 2003, Mantovani 1999, Rollins 1997).

1.5.2 Biological functions of chemokines

Chemokines are produced by the majority of somatic cells including monocytes/macrophages, ECs, SMCs and T-cells in the atherosclerotic lesion. They are mostly secreted although there are two membrane bound chemokines; fractalkine (CX3CL1) and CXCL16. Chemokine signalling is mediated through binding to 7-transmembrane G-coupled receptors. Downstream signalling cascades are usually calcium-dependent and often involve the activation of PI3K (Curnock *et al.* 2002, Sozzani *et al.* 1993). Chemokine receptors are classified according to the class of ligand to which they bind and so fall into CC, CXC, CX3C and C receptor families (IUIS/WHO Subcommitte on Chemokine Nomenclature 2003, Murphy 2002). Each receptor may be activated by a number of different ligands (see Table 1.5).

Table 1.5 Human chemokine receptors and their ligands

Abbreviations: BCA-1, B lymphocyte chemoattractant; CTACK, cutaneous T-cell-attracting chemokine; DC, dendritic cell; EC, endothelial cell; ENA-78, epithelial neutrophil-activating peptide 78; GCP-2, granulocyte chemotactic protein-2; GRO, growth related oncogene; HCC, human CC chemokine; IL-8, interleukin 8; IP-10, interferon-γ-inducible protein 10; I-TAC, interferon-I inducible T-cell α chemoattractant; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MEC, mammary-enriched chemokine; Mig, monokine induced by interferon γ; MIP, macrophage inflammatory protein; MPIF-1, myeloid progenitor inhibitory factor 1; NAP-2, neutrophil-activating peptide 2; NK cell, natural killer cell; PMN, polymorphonuclear leukocyte (eosinphil, neutrophil, basophil, mast cell); RANTES, regulated on activation normal T cell-expressed and secreted; SCM-1β, single cystein motif-1β; SDF-1, stromal-cell-derived factor 1; SLC, secondary lymphoid tissue chemokine; SMC, smooth muscle cell; TARC, thymus and activation-regulated chemokine; TECK, thymus-expressed chemokine (adapted from Lucas and Greaves 2001, Mantovani 1999).

RECEPTOR	LIGAND		CELLULAR RECEPTOR EXPRESSION				
	'OLD' NAME	STANDARD NOMENCLATURE	PMN	MONOCYTE/ MACROPHAGE	NK CELL	T CELL	OTHER
CC family							
CCR1	MIP-1α MIP-1β RANTES MCP-3 HCC-1 HCC-2 HCC-4 MPIF-1	CCL3 CCL4 CCL5 CCL7 CCL14 CCL15 CCL16 CCL23	+	+	+	+	DC B cell SMC
CCR2	MCP-1 MCP-3 MCP-2 MCP-4 HCC-4	CCL2 CCL7 CCL8 CCL13 CCL16	+	+	+	+	DC EC Fibroblast SMC
CCR3	RANTES MCP-3 MCP-2 Eotaxin MCP-4 HCC-2 Eotaxin-2 Eotaxin-3 MEC	CCL5 CCL7 CCL8 CCL11 CCL13 CCL15 CCL24 CCL26 CCL28	+			+	Epithelial cell
CCR4	TARC MDC	CCL17 CCL22		+		+	DC
CCR5	MIP-1α MIP-1β RANTES HCC-1	CCL3 CCL4 CCL5 CCL14		+	+	+	DC
CCR6	MIP-3α	CCL20	+		+	+	DC B cell
CCR7	MIP-3β SLC	CCL19 CCL21			+	+	DC B cell
CCR8	I-309	CCL1	+	+		+	
CCR9	TECK	CCL25				+	
CCR10	CTACK MEC	CCL27 CCL28		+		+	B cell
CXC family							
CXCR1	GCP-2 IL-8	CXCL6 CXCL8	+	+			EC
CXCR2	GRO-α GRO-β GRO-γ ENA-78 GCP-2 NAP-2 IL-8	CXCL1 CXCL2 CXCL3 CXCL5 CXCL6 CXCL7 CXCL8	+	+			EC
CXCR3	Mig IP-10 I-TAC	CXCL9 CXCL10 CXCL11	+		+	+	EC
CXCR4	SDF-1	CXCL12	+	+	+	+	DC
CXCR5	BCA-1	CXCL13		+		+	B cell
CXCR6		CXCL16		+		+	
CX3C family							
CX3CR1	Fractalkine	CX3CL1		+	+		SMC
C family							
XCR1	Lymphotactin SCM-1β	XCL1 XCL2				+	

Traditionally, chemokines have been viewed as effectors of immune cell function, however receptors are also present on other cell types including ECs and SMCs (see Table 1.5) (Mantovani 1999, Rollins 1997). Similarly, while research has focused on the role of chemokines in immune cell recruitment in inflammation, it is now recognised that they also have important functions in the regulation of cellular state (*e.g.* proliferation, activation, differentiation), discussed separately below.

1.5.2.1 Role of chemokines in immune cell recruitment

Leukocyte extravasation occurs in three stages: leukocyte rolling; firm adhesion to the endothelium; and migration into the arterial intima. At the site of inflammation chemokines present at the endothelial surface provide a cue for the firm binding of leukocytes and a chemokine gradient spanning the endothelium directs extravasation into the intima. Chemokines both upregulate the expression of adhesion molecules on the surface of the endothelium and promote the activation of integrins to increase binding affinity. They also stimulate the secretion of other inflammatory mediators to further increase immune cell recruitment (Cybulsky *et al.* 2004, Montavani 1999, Rollins 1997). The CX3C chemokine, fractalkine, is able to act as an adhesion molecule itself. Fractalkine is unusual in that the chemokine domain is anchored to the cell membrane by a mucin-rich stalk attached to a transmembrane domain. The chemokine is presented on the surface of ECs in inflammation and mediates the adhesion of monocytes, T-lymphocytes, NK cells and dendritic cells (DCs) by interacting with CX3CR1 on the target cell. The chemokine domain can also be shed through proteolytic cleavage by the enzymes ADAM17 (TNF-α converting enzyme) or ADAM10 (disintegrin-like MMP). The secreted chemokine can induce chemotaxis, activate monocytes and enhance the binding affinity of integrins (Haskell *et al.* 1999, Fong *et al.* 1998, Bazan *et al.* 1997, Imai *et al.* 1997).

1.5.2.2 Role of chemokines in the regulation of cellular state

Chemokines have a role in the regulation of both immune cell activation and cellular proliferation in inflammation (Mantovani 1999, Rollins 1997). Enhanced proliferation of ECs in response to a subset of chemokines stimulates angiogenesis.

The family of CXC chemokines is further divided into two groups based on the presence or absence of an ELR (glutamate-leucine-arginine) motif in the N-terminal domain. ELR-containing chemokines (*e.g.* IL-8, ENA-78 and GCP-2) are generally angiogenic while non-ELR CXC chemokines (*e.g.* IP-10 and Mig) are found to inhibit angiogenesis (Strieter *et al.* 1995). Some CC chemokines also stimulate angiogenesis including MCP-1 and eotaxin (Salcedo *et al.* 2001, Salcedo *et al.* 2000, Weber *et al.* 1999a). Chemokines have a role in hematopoiesis (the generation of new blood cells from myeloid progenitors) through the inhibition of proliferation, regulation of survival and promotion of chemotaxis. A large number of chemokines have the ability to suppress the proliferation of progenitor cells including MIP-1α, GRO-β, GRO-γ, PF4, IL-8 and MCP-1. Those that do not exert this effect, and in some cases counteract the suppressive effect of other chemokines, include MIP-1β, GRO-α, GRO-γ and RANTES (Broxmeyer 2001, Youn et 2000, Broxmeyer and Kim 1999).

1.5.3 Chemokines and atherosclerosis

As an inflammatory disease, the overall expression of chemokines in the atherosclerotic lesion is high, stimulated by the cocktail of cytokines and oxidised lipids present. The role of chemokines in the pathogenesis of atherosclerosis is currently of particular interest. A number of studies have suggested a pro-atherogenic impact and broad-spectrum chemokine inhibitors offer a promising thereapeutic strategy for the treatment of the disease (Grainger and Reckless 2003, Johnson *et al.* 2004). Several recent reviews discuss the various functions of chemokines in atherosclerosis in detail (Quehenberger 2005, Charo and Taubman 2004, Sheikine and Hansson 2004, Burke-Gaffney *et al.* 2002, Lucas and Greaves 2001, Reape and Groot 1999) and the effects are summarised in Table 1.6.

Particular interest in the role of chemokines in atherosclerosis has been generated due to important studies involving the CC chemokine MCP-1, which have shown a beneficial effect to deficiency in either the chemokine or its receptor CCR2 in mouse models of atherosclerosis (Dawson *et al.* 1999, Gosling *et al.* 1999, Boring *et al.* 1998, Gu *et al.* 1998). This chemokine is discussed in detail in Section 1.6. Other chemokines that have been shown to be present in the atheroma, in either humans

or mouse models of the disease, are listed in Table 1.6. Each chemokine displays a differential pattern of expression and localisation within the lesion and this is likely to vary over the course of the disease.

Knock-out mouse models (summarised in Table 1.6) have revealed the function and importance of specific chemokines and receptors in atherosclerosis and also the degree of functional redundancy in the chemokine system. For instance, the receptors CCR1 and CCR5 both bind to the chemokines RANTES, MIP-1α and MIP-1β, all of which have been localised to the lesion (Pattison *et al.* 1996, Wilcox *et al.* 1994). While knock-out of the CCR5 receptor in apoE-null mice does not confer an atheroprotective effect (Kuziel *et al.* 2003), blocking both CCR1 and CCR5 signalling using the inhibitor Met-RANTES decreases atherosclerosis development in apoE- and LDL-R-deficient mice (Veillard *et al.* 2004, Schober *et al.* 2002). It is also noticeable that while MCP-1- or CCR2-deficiency in mouse models of the disease significantly reduces the number of monocytes/macrophages within the lesion, these cells are still present, indicating the existence of alternative mechanisms of recruitment (Dawson *et al.* 1999, Gosling *et al.* 1999, Boring *et al.* 1998, Gu *et al.* 1998).

The membrane bound chemokines fractalkine and CXCL16 are of particular interest in atherosclerosis. As discussed previously, in addition to its role as a chemoattractant, fractalkine can also act as a cellular adhesion molecule in the recruitment of monocytes/macrophages and T-cells. A knock-out mouse model deficient in fractalkine and apoE has recently been generated that exhibits reduced atherosclerosis in comparison to the apoE-null mouse (Teupser *et al.* 2004). Mice lacking the receptor CX3CR display a similar phenotype (Combadiere *et al.* 2003, Lesnik *et al.* 2003). The chemokine CXCL16 has similar domain organisation to fractalkine; including a chemokine domain anchored to the membrane by a mucin-rich stalk, which may be released by enzymatic cleavage (Wilbanks *et al.* 2001). CXCL16 also has a dual role in atherosclerosis, functioning as both a chemoattractant for activated T-cells, and as a scavenger receptor contributing to the uptake of oxLDL in macrophage-foam cell formation (Wuttge *et al.* 2004, Minami *et al.* 2001).

Table 1.6 Chemokines in the atherosclerotic lesion

CHEMOKINE	FUNCTIONS IN ATHEROSCLEROSIS	REFERENCES[1]	
		LOCALISATION TO LESION	KNOCK-OUT MOUSE MODELS
MCP-1	Monocyte chemoattractant T-cell chemoattractant Adhesion of leukocytes to EC SMC migration and proliferation Angiogenesis	Rayner *et al.* 2000 Seino *et al.* 1995 Takeya *et al.* 1993	Gu *et al.* 1998 (MCP -/-, LDLR -/-) Boring *et al.* 1998 (CCR2-/-, apoE -/-) Gosling *et al.* 1999 (MCP -/-, overexpression apoB) Aiello *et al.* 1999 (Overexpression MCP-1 BMT, apoE -/-) Dawson *et al.* 1999 (CCR2-/-, apoE -/-)
MCP-4	Monocyte chemoattractant Activated T-cell chemoattractant	Berkhout *et al.* 1997	
MIP-1α	Monocyte chemoattractant Activated T-cell chemoattractant	Wilcox *et al.* 1994	Schober *et al.* 2002 (Inhibition CCR1/5 signalling with Met-RANTES, apoE-/-)
MIP-1β	Monocyte chemoattractant T-cell chemoattractant		Veillard *et al.* 2004 (Inhibition CCR1/5 signalling with Met-RANTES, LDLR-/-)
RANTES	Monocyte chemoattractant Activated T-cell chemoattractant Activation of macrophages Activation of T-cells	Pattison *et al.* 1996 Wilcox *et al.* 1994	
PARC	Naïve T-cell chemoattractant	Reape *et al.* 1999	
MIP-3β	T-cell chemoattractant Adhesion of T-cells to EC		
TARC	Activated T-cell chemoattractant Platelet activation and aggregation	Greaves *et al.* 2001	
MDC	Activated T-cell chemoattractant Platelet activation and aggregation		
IL-8	Monocyte chemoattractant Activated T-cell chemoattractant Angiogenesis SMC migration and proliferation Adhesion of monocytes to EC	Boisvert *et al.* 1998 (CXCR2) Wang *et al.* 1996a Apostolopoulos *et al.* 1996	Boisvert *et al.* 1998 (CXCR2-/- BMT, LDLR-/-)
GRO-α	Monocyte chemoattractant Activated T-cell chemoattractant Angiogenesis SMC migration and proliferation	Boisvert *et al.* 1998 (CXCR2)	
CXCL16	Scavenger receptor Activated T-cell chemoattractant	Minami *et al.* 2001 Wuttge *et al.* 2004	
Eotaxin	SMC migration	Haley *et al.* 2000	Veillard *et al.* 2005 (CXCR3-/-, apoE-/-)
Fractalkine	Cell-adhesion molecule Monocyte chemoattractant T-cell chemoattractant Adhesion of monocytes to EC SMC migration Platelet activation	Wong *et al.* 2002 Lucas *et al.* 2003	Teupser *et al.* 2004 (Fractalkine -/-, apoE-/- and LDLR-/-) Lesnik *et al.* 2003 (CXC3R-/-, apoE-/-) Combadiere *et al.* 2003 (CXC3R-/-, apoE-/-)
IP-10	Activated T-cell chemoattractant SMC migration	Mach *et al.* 1999	
Mig	Activated T-cell chemoattractant		
ITAC	Activated T-cell chemoattractant		
SDF-1α	T-cell chemoattractant Platelet activation and aggregation	Abi-Younes *et al.* 2000	

In general, chemokines increase the cellularity of the lesion through: increased recruitment of monocytes/macrophages, T-cells and SMCs; enhanced proliferation of SMCs; immune cell activation; and stimulation of the secretion of proinflammatory mediators. In inflammation and atherosclerosis, the recruitment of monocytes/macrophages appears to be largely regulated by the CC chemokines (*e.g.* MCP-1 and -4, MIP-1α and -1β, RANTES) although CXC chemokines such as IL-8 and the CX3C chemokine fractalkine have also been shown to recruit monocytes. The CXC chemokines predominantly recruit activated T-cells (*e.g.* IL-8, GRO-α, IP-10, Mig, ITAC) (Quehenberger 2005, Charo and Taubman 2004, Sheikine and Hansson 2004, Burke-Gaffney *et al.* 2002, Lucas and Greaves 2001, Reape and Groot 1999). The CC chemokine PARC preferentially attracts naïve T-cells (Adema *et al.* 1997) that are activated within the lesion by inflammatory cytokines and chemokines such as the CC-chemokines RANTES, MIP-1α and MCP-1 (Taub *et al.* 1996, Bacon *et al.* 1995). The chemokine receptors CCR1, CCR2, CCR3, CCR5, CXCR4 and CX3CR have been shown to be present on the surface of SMCs and the migration and proliferation of these cells can be regulated by chemokines in the lesion (*e.g.* IP-10, IL-8, MCP-1, fractalkine, eotaxin) (Kodali *et al.* 2004, Lucas *et al.* 2003, Schecter *et al.* 2003, Schecter *et al.* 2000, Hayes *et al.* 1998, Wang *et al.* 1996b). Eotaxin is known primarily for the recruitment of eosinophils but is associated with SMC-rich areas of the atherosclerotic plaque and is thought to function in the recruitment of these cells in the disease (Kodali *et al.* 2004, Haley *et al.* 2000).

In the advanced plaque chemokines are likely to have a role in neovascularisation. As previously discussed, ELR-containg CXC chemokines (*e.g.* IL-8) and CC chemokines (*e.g.* MCP-1) stimulate angiogenesis. Non-ELR CXC chemokines (*e.g.* IP-10, Mig) however inhibit this process so that the overall role of chemokines in neovascularisation is uncertain (Strieter *et al.* 1995). Additionally, particular chemokines increase the thrombogenic potential of the plaque as they are known to cause platelet activation and clotting. These include SDF, TARC, MDF and fractalkine (Schafer *et al.* 2004, Abi-Younes *et al.* 2001, Abi-Younes *et al.* 2000).

1.6 MONOCYTE CHEMOATTRACTANT PROTEIN-1

Of the chemokines discussed above, the CC chemokine MCP (monocyte chemoattractant protein)-1 (CCL2) has attracted the most interest in relation to its role in atherosclerosis and has been shown in several independent studies to be a proatherogenic factor (Harrington 2000, Rollins 1996). The regulation of MCP-1 is of particular interest as it presents a potential therapeutic target for atherosclerosis. Transfection of a N-terminal deletion mutant of the MCP-1 gene (7ND) in apoE-null mice has been shown to inhibit both lesion formation and the progression of existing lesions, as well as increasing plaque stability (Inoue *et al.* 2002, Ni *et al.* 2001).

1.6.1 Biological roles of MCP-1

There are four functional MCP chemokines in humans (MCP 1-4) and three in mice (MCP 1, 3 and 5) (Berkhout *et al.* 1997, Sarafi *et al.* 1997, Proost *et al.* 1996). A variety of cell types produce MCP-1 including *monocytes/macrophages*, ECs, VSMCs, fibroblasts, chondrocytes, astrocytes and epithelial cells as well as certain cancerous cells (Proost *et al.* 1998, Zhang *et al.* 1996). MCP-1 functions as a dimer and is structurally similar to MIP-1β. The murine protein (also known as JE) shares considerable sequence homology with human MCP-1 but has an additional O-linked carbohydrate at the C-terminus, the function of which is unclear (Lubkowski *et al.* 1997, Zhang *et al.* 1996a). The action of MCP-1 is mediated by binding to the G-protein linked CC chemokine receptor, CCR2, on the cell surface (Section 1.5) (IUIS/WHO Subcommitte on Chemokine Nomenclature 2003, Boring *et al.* 1997).

The major biological function of MCP-1 is to promote the chemotaxis of monocytes to the site of inflammation, although it has also been shown to be involved in the recruitment of T-lymphocytes, NK cells and in the migration of VMSCs (Proost *et al.* 1998). Other actions of this chemokine include: increasing monocyte adhesion to the endothelium through the activation of cell adhesion molecules (Gerszten 1999); modulating Th1 and Th2 responses and the expression of inflammatory mediators (*e.g.* IFN-γ, IL-4 and IL-5) (Gu *et al.* 2000, Peters *et al.* 2000, Lu *et al.* 1998);

increasing the proliferation SMCs (Seltzman *et al.* 2002); and stimulation of angiogenesis (Salcedo *et al.* 2001, Weber *et al.* 1999a). Knock-out mice deficient in MCP-1 show an impaired ability to recruit monocytes in models of inflammation and exhibit decreased expression of cytokines including IL-4, IL-5 and IFN-γ. Other responses are unaffected in these mice such as the ability to clear infection with *Mycobacterium tuberculosis* (Lu *et al.* 1998). Deletion of the CCR2 gene (that also functions as receptor for MCP-2, -3, -4 and HCC-4) in mice leads to similar defects in monocyte/macrophage recruitment in response to inflammatory stimuli. These mice exhibited a dramatic reduction in the production of IFN-γ as well as the cytokines IL-4, IL-5 and IL-12 (Boring *et al.* 1997).

MCP-1 has been linked to several pathological processes characterised by the infiltration of monocytes, including: inflammatory skin diseases (e.g. cutaneous sclerosis) (Yamamoto 2003); allergic asthma (Ross *et al.* 2003); autoimmune diseases such as multiple sclerosis (Mahad and Ransohoff 2003, Simpson *et al.* 1998) and autoimmune encephalomyelitis (Mahad and Ransohoff 2003, Ransofhoff *et al.* 1993), as well as atherosclerosis.

1.6.2 Role of MCP-1 in atherosclerosis

A proatherogenic role has been well established for MCP-1, characterised predominantly by an increased accumulation of macrophage- and SMC-derived foam cells (Harrington 2000, Rollins 1996). As well as the role of MCP-1 in the chemoattraction of monocytes/macrophages, T-cells and VSMCs, the pro-inflammatory effects on the expression of other cytokines and enhanced angiogenesis stimulated by the chemokine, all contribute to atherogenesis and destablisation of the plaque. High levels of expression of MCP-1 have been detected in the atherosclerotic lesion by antibody detection and *in situ* hybridisation, and elevated levels have been found in patients in association with risk factors for atherosclerosis (de Lemos *et al.* 2003, Rayner *et al.* 2000, Seino *et al.* 1995, Takeya *et al.* 1993). Knock-out of the MCP-1 gene in apoE- and LDL-R-null mice significantly reduces cellularity and lesion area without affecting plasma lipid or lipoprotein levels (Gosling *et al.* 1999, Gu *et al.* 1998). Conversely increased expression of MCP-1 enhances atherosclerosis formation in apoE-null

mice (Aiello *et al.* 1999). Local overexpression of MCP-1 at the blood vessel wall in rabbits was not sufficient by itself to induce atherosclerosis formation but acted in synergy with hypercholesterolemia to promote atherosclerotic changes (Namiki *et al.* 2002). Knock-out of the MCP-1 receptor, CCR2 in apoE-null mice has a similarly atheroprotective effect (Dawson *et al.* 1999, Boring *et al.* 1998). Expression of CCR2 on monocytes has been found to be increased in patients with hypercholesterolemia and is upregulated by high levels of LDL (Han and Quehenberger 2000, Han *et al.* 1999, Han *et al.* 1998). Studies have shown however that, in contrast to native LDL, oxLDL has an inhibitory effect on monocyte CCR2 expression (Han *et al.* 2000, Han *et al.* 1998). This has been explained by a model in which monocytes are recruited to the lesion through CCR2 signalling and retained at the site by downregulation of CCR2 in the presence of high levels of oxidised lipid. Another study has demonstrated the opposite effect, whereby oxLDL increased the expression of CCR2 in monocytes (Weber *et al.* 1999b).

1.6.3 Regulation of MCP-1 expression by inflammatory mediators

A number of inflammatory cytokines regulate the expression of MCP-1 in a variety of cells types, including IFN-γ, IL-1β, IL-4, TNF-α , TGF-β, GM-CSF and PDGF (reviewed in Proost *et al.* 1998). Proinflammatory cytokines generally induce MCP-1 expression (*e.g.* IFN-γ (Valente *et al.* 1998), IL-1β and TNF-α (Bian *et al.* 2004)) while antiinflammatory cytokines suppress MCP-1 gene expression (*e.g.* IL-10 and IL-13 (Kucharzik *et al.* 1998)). For some cytokines there is conflicting data about the regulatory role in MCP-1 expression and this is potentially dependent on cell type (*e.g.* TGF-β upregulates MCP-1 in ECs and mesangial cells (Cheng *et al.* 2005, Matagrano *et al.* 2004) but downregulates expression in macrophages (Feinberg *et al.* 2004); IL-4 induces expression in ECs (Lee *et al.* 2003) but downregulates MCP-1 in epithelial cells (Kucharzik *et al.* 1998)).

Transcriptional regulation of human MCP-1 appears to be controlled largely through specific distal (-2500/-2300) and proximal promoter regions (-300/+1). Binding sites for NF-κB have been identified in both regions while the proximal region also contains a putative GAS site, a GC-box, AP-1 (TPA-responsive element (TRE)) sites and possible C/EBP binding sites. Inducible and constitutive binding to these sites

has been demonstrated in various combinations in different cell lines and in response to particular mediators, as summarised in Table 1.7. Shear stress, thought to promote atherosclerotic plaque formation at particular arterial sites, has also been demonstrated to either stimulate the production of MCP-1 by ECs (Bao *et al.* 1999, Shyy *et al.* 1995) or to downregulate MCP-1 gene expression (Chiu *et al.* 2002), potentially in response to varying flow forms (Vanderlaan *et al.* 2004, Bao *et al.* 1999).

Table. 1.7 Regulation of MCP-1 gene expression; promoter binding studies[1]

MEDIATOR (+/-)	CELL TYPE	PROMOTER SITE	SPECIES	REFERENCE
IL-1β (+)	Endothelial	AP-1	Human	Bian *et al.* 2004
	Endothelial	NFκB	Human	Choi *et al.* 2004
	Mast	NFκB	Human	Lee *et al.* 2004
	Endothelial	AP-1 (-68), NFκB (-90)	Human	Martin *et al.* 1997
IFN-γ (+)	Astrocyte	STAT1 (-212), Sp1 (-124)	Human	Zhou *et al.* 2001
	Astrocyte	STAT1 (-212), Sp1 (-124)	Human	Zhou *et al.* 1998
	Osteoblast	STAT1 (-212), IRIS [2] (-227)	Human	Valente *et al.* 1998
IL-4 (+)	Endothelial	STAT1 (-212)	Human	Lee *et al.* 2003
Insulin (+)	VSMC	C/EBP-β (-2589, -3116)	Rat	Sekine *et al.* 2002
	VSMC	C/EBP-β (-2589, -3116)	Rat	Kodama *et al.* 2005
Glucose (+)	Mesangial	AP-1	Human	Lee *et al.* 2001
LPS (+)	Macrophage	C/EBP-β	Mouse	Hu *et al.* 2000
	Macrophage	κB3 binding site (-169)	Mouse	Ueno *et al.* 2000
	Epithelial	NFκB(-2287,-2261), AP-1(-54)/Sp1(-51)	Rat	Wang *et al.* 2000b
	Macrophage	C/EBP-β	Mouse	Bretz *et al.* 1994
PDGF BB (+)	Fibroblast	Sp1, Sp3 (-51)	Mouse	Ping *et al.* 1999
	Fibroblast	Sp3 [2] (-1468, -1434)	Mouse	Sridhar *et al.* 1999
ROS (+)	Endothelial	NFkB, AP-1	Dog	Lakshminarayanan *et al.* 2001
Shear stress (+)	Endothelial	NFκB	Human	Bao *et al.* 1999
	Endothelial	AP-1 (-60)	Human	Shyy *et al.* 1995
Shear stress (-)	Endothelial	AP-1	Human	Chiu *et al.* 2002
Tat (HIV) (+)	Astrocyte	Sp1(-124), AP-1(-128), NFκB(-150)	Human	Lim and Garzino-Demo 2000
	Astrocyte	C/EBP-β (0//-200), Smad3 [2]	Human	Abraham *et al.* 2005
TGF-β (-)	Macrophage	AP-1, Smad3 [2] (-122 human)	Mouse	Feinberg *et al.* 2004
TNF-α (+)	Endothelial	AP-1	Human	Bian *et al.* 2004
	Fibroblast	NFκB (-2378, 2352), Sp1 (-51)	Mouse	Boekhoudt *et al.* 2003
	Endothelial	NFκB (-2378, 2352), Sp1 (-51)	Mouse	Ping *et al.* 2000
	Mesangial	NFκB (-2378, 2352), Sp1 (-51)	Mouse	Ping *et al.* 1999
	Fibroblast	NFκB (-2378, 2352), Sp1 (-51)	Mouse	Ping *et al.* 1996

[1] Where transcription factor binding has been localised to a particular site in the MCP-1 promoter, the position of the site upstream of the transcriptional start site (+1) is given in parentheses. In other cases increased DNA binding has been shown to a consensus element for that transcription factor.

[2] Transcriptional repression

Abbreviations: AP-1, activating protein-1; C/EBP-β, CCAAT/enhancer binding protein-β; HIV, human immunodeficiency virus; IFN-γ, interferon-γ, IL-1β, interleukin-1β; LPS, lipopolysaccharide; NFκB, nuclear factor κB; PDGF BB, platelet-derived growth factor BB; ROS, reactive oxygen species; STAT1, signal transducer and activator of transcription 1; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

Although studies in astrocytes have demonstrated the presence of a functional STAT1-binding site, that is involved in promoter activation by IFN-γ (Table 1.7), a study of gene expression in STAT1-null bone marrow-derived macrophages (BMMs) showed MCP-1 induction by IFN-γ in both wild type and STAT1-deficient cells, suggesting that a STAT1-independent pathway may also exist (Gil *et al.* 2001, Ramana *et al.* 2002). In the following sections potential STAT1-dependent and -independent mechanisms of IFN-γ signalling are discussed.

1.7 ROLE OF THE JAK-STAT PATHWAY IN IFN-γ SIGNALLING

Cytokines mediate signalling through binding to specific receptors at the cell surface. The association of the cytokine with its receptor generally leads to alterations in receptor confirmation and initiates a specific signalling cascade resulting in changes in transcription, translation or post-translational modifications. The key components of the majority of signalling pathways are protein kinases, which control the activation of downstream effectors through the phosphorylation of particular residues.

1.7.1 JAK-STAT signalling

JAK-STAT signalling is involved in the mediation of responses to a number of cytokines (*e.g.* IFN-α, IFN-β, IFN-γ, IL-4, IL-6, IL-13). JAKs (including tyrosine kinase 2 (Tyk2) and JAK1-3) are receptor-associated tyrosine kinases that are activated by cytokine binding to the receptor. STATs (including STAT1-4, -5a, -5b and -6) are SH2-domain containing transcription factors that bind to DNA as a homo- or heterodimer. Various JAK-STAT components are associated with the mediation of signalling in response to different cytokines. For instance: IL-6 treatment can lead to the activation of JAK1, JAK2 or Tyk2 and STAT1 and STAT3; Type 1 IFNs activate Tyk2, JAK1, STAT1 and STAT2 (that also associate with IRF-9 to form the transcription factor ISGF3); while IFN-γ signalling involves JAK1, JAK2 and STAT1 (Kerr *et al.* 2003, Aaronson and Horvath 2002, Schindler and Darnell 1995). The "JAK-STAT pathway", as associated with IFN-γ signalling, has been extensively studied and is discussed in detail below.

1.7.2 JAK-STAT signalling in the IFN-γ response

The cellular response to IFN-γ is mediated via interaction of the cytokine with its receptor, IFN-γR. IFN-γR is a heterodimeric receptor with α and β chains (IFN-γR1 and IFN-γR2), both of which are required in order for the receptor to function in the transduction of the IFN-γ signal (Tau and Rothman 1999, Marsters *et al.* 1995).

The most widely accepted signalling mechanism for the mediation of the IFN-γ response is the JAK-STAT pathway, elucidated through studies of mutant cell lines lacking specific components of the pathway (reviewed in Stark *et al.* 1998, Schindler and Darnell 1995). JAK1 is bound to the intracellular domain of IFN-γR1 while JAK2 binds IFN-γR2. In the absence of IFN-γ stimulation, receptor subunits are not strongly associated with one another. Ligand binding to IFN-γR causes assembly of the active receptor complex in which the intracellular domains of the receptor subunits are brought together. This allows activation of JAK1 and JAK2 by auto- and *trans*-phosphorylation. Subsequent phosphorylation of a specific tyrosine containing sequence on the IFN-γR1 subunit provides a docking site for STAT1 through a SH2 domain. Receptor associated STAT1 is phosphorylated by the JAK proteins (at tyrosine 701) causing it to dissociate from the receptor and form homodimers. Phosphorylation of STAT1 at Serine 727 is also required for maximal activation although the mechanisms involved in this are less clear (Decker and Kovarik 2000, Wen *et al.* 1995).

STAT1 homodimers translocate to the nucleus and regulate transcription of the majority of IFN-γ-responsive genes through gamma-activated sequence (GAS) promoter elements (consensus sequence: TTNCNNNAA). However other elements have also been found to be necessary for the transcription of certain genes by IFN-γ, including the interferon-stimulated response element (ISRE) (more commonly associated with Type I IFN-signalling), palindrome IFN-response element (pIRE) and gamma-IFN-activated transcriptional element (GATE). The signalling mechanisms involved in the JAK-STAT pathway are reviewed in a number of articles (Kalvakolanu 2003, Kerr *et al.* 2003, Aaronson and Horvath 2002, Stark *et al.* 1998, Schindler and Darnell 1995) and illustrated in Figure 1.2.

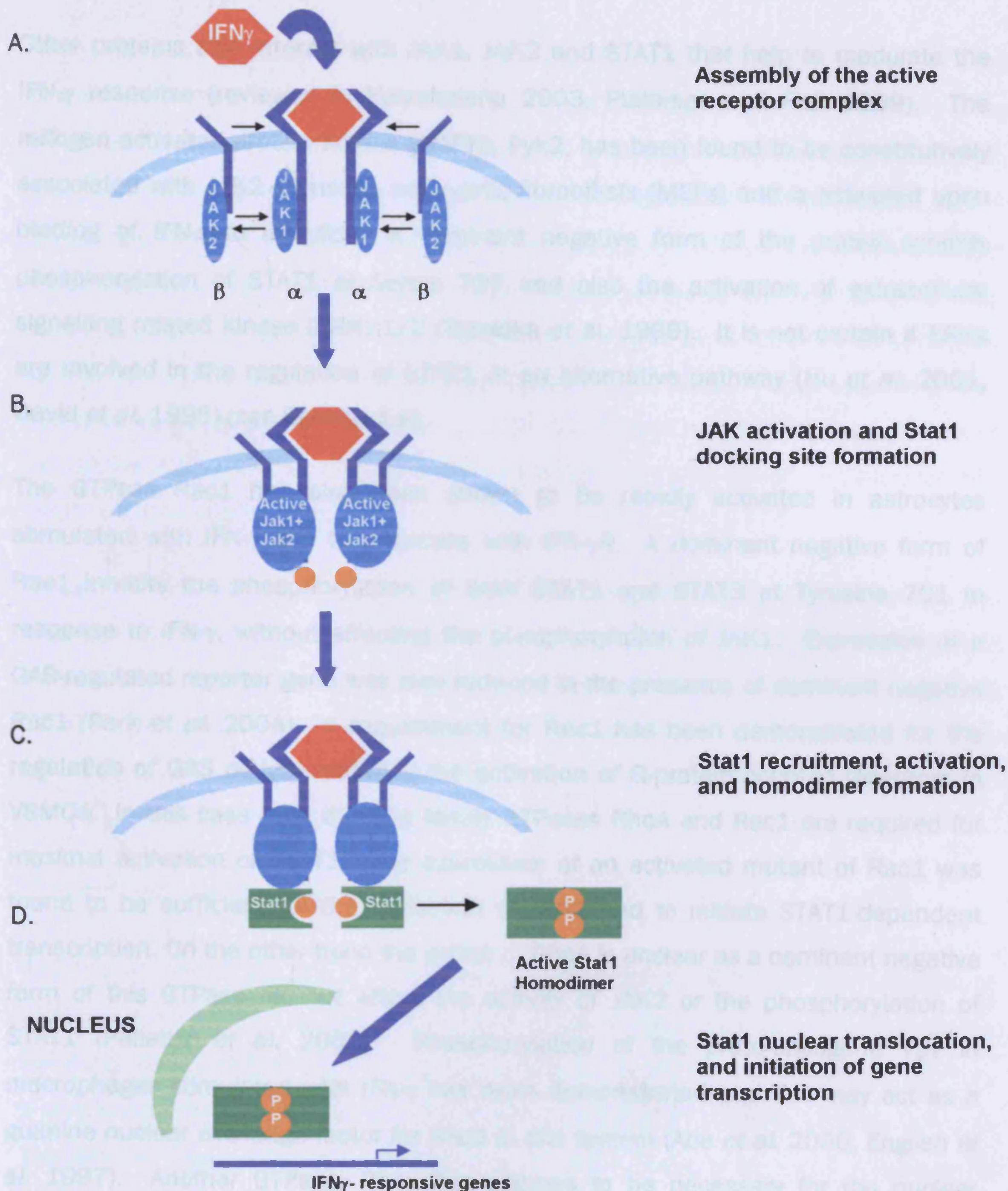


Figure 1.2 The JAK-STAT pathway

A. Binding of IFN-γ to IFN-γR1 causes assembly of the receptor complex and auto- and trans-phosphorylation of JAK1 and JAK2.
B. Activated JAKs phosphorylate IFN-γR1 to provide a docking site for STAT1.
C. STAT1 is recruited to the active receptor complex where it is activated and forms a homodimer.
D. The STAT1 homodimer translocates to the nucleus and binds to GAS elements in the promoter of IFN-γ responsive genes to initiate transcription (adapted from Stark *et al.* 1998).

Abbreviations: IFN-γ, interferon-γ; JAK, Janus kinase; P, phosphorylated residue; STAT1, signal transducer and activator of transcription.

Other proteins also interact with JAK1, JAK2 and STAT1 that help to modulate the IFN-γ response (reviewed in Kalvakolanu 2003, Platanias and Fish 1999). The mitogen activated protein kinase (MAPK), Pyk2, has been found to be constitutively associated with JAK2 in mouse embryonic fibroblasts (MEFs) and is activated upon binding of IFN-γ to IFN-γR1. A dominant negative form of the protein inhibits phosphorylation of STAT1 at Serine 727 and also the activation of extracellular signalling related kinase (ERK) 1/2 (Takaoka *et al.* 1999). It is not certain if ERKs are involved in the regulation of STAT1 or an alternative pathway (Hu *et al.* 2001, David *et al.* 1995) (see Section 1.8).

The GTPase Rac1 has also been shown to be rapidly activated in astrocytes stimulated with IFN-γ and to associate with IFN-γR. A dominant negative form of Rac1 inhibits the phosphorylation of both STAT1 and STAT3 at Tyrosine 701 in response to IFN-γ, without affecting the phosphorylation of JAK1. Expression of a GAS-regulated reporter gene was also reduced in the presence of dominant negative Rac1 (Park *et al.* 2004). A requirement for Rac1 has been demonstrated for the regulation of GAS activity following the activation of G-protein coupled receptors in VSMCs. In this case both the Rho family GTPases RhoA and Rac1 are required for maximal activation of STAT1. The expression of an activated mutant of Rac1 was found to be sufficient for the activation of JAK2 and to initiate STAT1-dependent transcription. On the other hand the action of RhoA is unclear as a dominant negative form of this GTPase did not affect the activity of JAK2 or the phosphorylation of STAT1 (Pelletier *et al.* 2003). Phosphorylation of the proto-oncogene Vav in macrophages stimulated with IFN-γ has been demonstrated and Vav may act as a guanine nuclear exchange factor for Rac1 in this system (Abe *et al.* 2000, English *et al.* 1997). Another GTPase, Ran, also appears to be necessary for the nuclear translocation of STAT1 in response to IFN-γ (Sekimoto *et al.* 1996).

The SH2-domain containing tyrosine phosphatases (SHP)-1 and SHP-2 both contribute to the regulation of STAT1 activity. These proteins are potentially recruited to IFN-γR through the SH2-domain, allowing the regulation of both JAK and STAT1 phosphorylation. The two phosphatases appear to have opposing effects whereby SHP-1 functions as a positive regulator of JAK-STAT signalling while SHP-2 has a negative role (You *et al.* 1999, You and Zhao 1997).

The expression of RNA-dependent protein kinase (PKR), activated by the accumulation of double-stranded RNA (dsRNA) in viral replication, is induced by IFN-γ and mediates many of the anti-viral and anti-proliferative actions of the cytokine. IFN-γ has also been shown to promote the phosphorylation of PKR and it may have a direct role in IFN-γ-induced signalling. Experiments in PKR-null MEFs indicate that PKR is necessary for the activation of the IRF1 promoter by the cytokine (Der *et al.* 1997, Kumar *et al.* 1997). Although the study by Kumar *et al.* (1997) did not support a role for STAT1 in the PKR-mediated regulation of IRF1, it has been found by Ramana *et al.* (2000), that in PKR-null cells, STAT1 was not properly phosphorylated on Serine 727 and that the expression of a reporter gene driven by GAS promoter elements was suppressed.

In the nucleus, several additional proteins associate with the STAT1 homodimer and regulate its transcriptional activity. These include CREB-binding protein (CBP/p300) (Zhang *et al.* 1996), mini chromosomal maintenance protein (MCM5) (Zhang *et al.* 1998b) and breast cancer susceptibility gene 1 (BRCA1) (Ouchi *et al.* 2000). The interaction of STAT1 with MCM5 and BRCA1 is thought to require the phosphorylation of STAT1 at Serine 727.

1.8 ALTERNATIVE IFN-γ SIGNALLING PATHWAYS

The importance of the JAK-STAT pathway in IFN-γ responses is clear from studies of STAT1-null mice and mice deficient in JAK1 or JAK2. Disruption of JAK genes causes perinatal (JAK1) and embryonic lethality (JAK2) while STAT1-knock-out mice develop a phenotype similar to those deficient in IFN-γ, showing an increased susceptibility to viral and microbial infections (Parganas *et al.* 1998, Rodig *et al.* 1998, Neubauer *et al.* 1998, Durbin *et al.* 1996, Meraz *et al.* 1996). However several recent studies have suggested the existence of alternative IFN-γ signalling pathways independent of JAK-STAT.

1.8.1 Regulation of gene expression by IFN-γ in STAT1-deficient cells

BMMs and MEFs deficient in STAT1 exhibit enhanced proliferation in response to IFN-γ while cell growth in wild type cells is inhibited. STAT1-null mice are also more resistant to infection with murine cytomegalovirus (MCMV) and Sindbis virus than

those lacking the IFN-γ receptor indicating the involvement of IFN-γ-dependent, STAT1-independent pathways in the response to these viruses. Analysis of gene expression in STAT1-deficient macrophages through representational difference analysis showed that IFN-γ induced the expression of certain genes, including MIP-1α, MIP-1β and MCP-1, and repressed that of others, such as CXCR4, in both wild type and STAT1-null cells. IL-1β and arginase genes were upregulated by IFN-γ only in cells lacking STAT1 (Gil *et al.* 2001). In these cases a STAT1-dependent pathway may be inhibiting gene expression while opposing, STAT1-independent, signals stimulate expression. MCP-1 was also found, by RNAase protection analysis, to be regulated in both STAT1-deficient and wild type fibroblasts although in this case MIP-1α was only induced in STAT1-null cells, while the induction of MIP-1β occurred only in wild type cells (Ramana *et al.* 2001). A study by Klampfer *et al.* (2004) found that siRNA against STAT1 inhibited the induction of certan genes by IFN-γ (*e.g.* IRF-1) but not that of others (*e.g.* caspase-7).

Microarray experiments have similarly indicated a large range of genes regulated independently of STAT1 (Table 1.8). IFN-γ was found to regulate 216 genes in wild type BMMs (187 upregulated and 29 downregulated) and 150 genes in STAT1-deficient BMMs (51 upregulated and 99 downregulated). As with IL-1β and arginase genes, a significant number of genes were differentially expressed in response to IFN-γ in STAT1-deficient cells only, and not in the wild type (Ramana *et al.* 2002, Gil *et al.* 2001). A parallel study in STAT1-null MEFs revealed 28 genes induced by more than 3.5 fold in response to IFN-γ, approximately a third of which were also regulated in wild type cells. These include several cell cycle regulating genes such as c-myc and c-jun that are suppressed in wild type fibroblasts and may be responsible for altered proliferative responses in these cells (Ramana *et al.* 2002, Ramana *et al.* 2001). The induction of c-myc expression in STAT1-null fibroblasts has been found to involve the oncogenic protein kinase Raf1 (Ramana *et al.* 2000). A study by Kortylewski *et al.* (2004) also implicated STAT1-independent pathways in altered growth responses to IFN-γ. Their results indicate that STAT1 is crucial for the antiproliferative effect of IFN-γ in tumor suppression and suggest the presence of alternative, STAT1-independent, pathways that counteract the inhibitory effects of IFN-γ on cell growth (Kortylewski *et al.* 2004).

Table 1.8 Genes regulated by IFN-γ in wild type and STAT1-null macophages

CELL TYPE	GENE	FOLD-CHANGE IN EXPRESSION	FUNCTION
Wild type	SOCS-1	123	Cytoplasimic; regulation
	MIP-1β	32	Secreted; chemokine
	MCP-1	22	Secreted; chemokine
	Zyxin	22	Cell motility
	MIG	20	Secreted; chemokine
	IP-10	20	Secreted; chemokine
	SOCS-3	13	Cytoplasmic regulation
	IRF-1	11	Transcription
	IRG-47	9.8	G-protein; regulation
	LRG-47	9.5	G-protein; regulation
	Ro RNP	9.4	Cell metabolism (suggested)
	p204	9.4	Cell-cycle regulation
	p203	9.0	Cell-cycle regulation
	MIP-1α receptor	8.7	Chemokine receptor
	GARG-39	7.6	Cell communication (suggested)
	Talin	7.1	Adhesion
	IGTP	6.7	Antimicrobial resistance
	TGTP	6.5	Antiviral resistance
	p202	6.4	Cell cycle regulation
	Tissue factor (mtf)	5.1	Coagulation
	TPL-2/Cot	4.7	Proto-oncogene; kinase
	Fibronectin	4.5	Extracellular matrix
	ICH-3	4.4	Apoptosis
	PIM-1	4.3	Proto-oncogene; cell proliferation
	GRO/KC	-21	Secreted; chemokine
	c-fos	-13	Oncogene; cell proliferation
	Mel-N1	-9.0	RNA-binding protein
	FAH	-6.7	Metabolism
	MKP-1	-5.5	Regulation; phosphatase
	MIP-2	-5.4	Secreted; chemokine
	TGIF	-4.4	Transcriptional co-repressor
	CXCR4	-4.0	Chemokine receptor
STAT1 -/-	MCP-1	20	Secreted; chemokine
	TDAG-51	12	Apoptosis
	Lice2 cysteine protease	7.6	Apoptosis
	IL-1β	6.9	Secreted; chemokine
	PRP-8	5.0	Splicing factor
	PIM-1	5.0	Proto-oncogene; cell proliferation
	Fibronectin	4.1	Extracellular matrix
	SOCS-3	4.0	Cytoplasmic; regulation
	A-X actin	-33	Cell motility; inhibits metastasis
	TI-227	-9.1	Metastasis
	Melanoma X actin	-8.0	Cell motility; inhibits metastasis
	MgluR8	-5.1	Receptor
	Zfx	-4.5	Transcription
	Rb	-4.2	Tumor suppressor

Soler *et al.* (2003) showed that nucleoside transporter activity is differentially regulated by IFN-γ in STAT1-null macrophages. STAT1 was required for the inhibiton of equilibrative nucleoside transporter (ENT) activity through posttranscriptional actions and for the inhibition of cell growth by IFN-γ. However, the activity and expression of concentrative nucleoside transporters (CNT) was regulated by IFN-γ independently of STAT1, as was the downregulation of ENT mRNA expression (Soler *et al.* 2003).

1.8.2 Potential pathways for STAT1-independent regulation of IFN-γ-modulated gene expression

Inhibition of JAK1 using a dominant negative mutant has shown certain antiviral responses that require the presence of functional JAK1 in addition to active STAT1, indicating that JAK1 has a role in the activation of other signalling molecules (Briscoe *et al.* 1996). Many genes regulated independently of STAT1 may still require the involvment of JAKs for IFN-γ-induced stimulation of transcription. In support of this hypothesis, the expression of MIP-1α, IL-1β and arginase genes (shown to be STAT1-independently regulated) was not induced by IFN-γ in JAK1-deficient BMMs (Gil *et al.* 2001).

Activated JAKs interact with a range of SH2 domain-containing proteins and IFN-γ-induced tyrosine phosphorylation at certain sites in the kinase domain may provide binding sites for other proteins. The Src-family kinase Fyn, for example, has been shown to bind to JAK2 via a SH2 domain, although the downstream targets of this kinase in IFN-γ-dependent signalling are unknown (Uddin *et al.* 1997). Other sites on IFN-γR are also phosphorylated by JAKs, in addition to the STAT1 binding site. These could provide docking sites for other signalling molecules (Ramana *et al.* 2002, Rane and Reddy 2000, Aringer *et al.* 1999). More detailed studies involving inhibition of JAK1 and JAK2 are required to fully understand the roles of these proteins in STAT-independent signalling.

The adapter molecules c-Cbl, CrkL, and CrkII are recruited by IFN-γR upon ligand binding. Alsayed *et al.* (2000) demonstrated that treatment of cells with IFN-γ leads

to tyrosine phosphorylation of c-Cbl, which in turn provides a binding site for CrkL. Phosphorylated CrkL then activates C3G, a guanine nucleotide exchange factor and Rap1. Rap1 is small G protein known to mediate some growth inhibitory responses. This pathway occurs in response to IFN-γ without the formation of CrkL-STAT complexes induced in IFN-α and β signalling, and so may be STAT-independent although receptor phosphorylation by JAK proteins may still be required (Alsayed *et al.* 2000, Platanias *et al.* 1999).

1.8.2.1 Transcription factors potentially involved in STAT1-independent signalling

1.8.2.1.1 NF-κB

The NF-κB family of transcription factors are dimeric complexes composed from a collection of subunits related by a conserved DNA-binding and dimerisation region, the Rel homology (RH) domain. Certain members are inactive (p105/50 and p100/52) and only function as transcriptional activators when they form heterodimers with proteins from a second subset (p65 (RelA), Rel (c-Rel) and RelB). The original NF-κB transcription factor characterised was the p50-p65 heterodimer. In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by an IκB inhibitory protein that blocks the nuclear localisation sequence. Activating stimuli cause the phosphorylation of an IκB kinase (IKK) complex (consisting of IKK-α, IKK-β and IKK-γ subunits) that specifically phosphorylates IκBs targeting them for ubiquitination and degradation. The removal of IκB exposes the nuclear localisation sequence and various phosphorylation sites required for the activation and translocation of NF-κB to the nucleus. Active NF-κB binds to a decameric DNA sequence (consensus sequence: GGG[A/G]NN[C/T][C/T]CC) in the promoter of target genes to regulate transcription (Delhalle *et al.* 2004, Li and Stark 2002).

Activation of NF-κB has been implicated in a number of inflammatory responses and it may be an important transcriptional regulator in atherosclerosis (Monaco and Paleolog 2004, Collins and Cybulsky 2001). NF-κB is activated by several proinflammatory cytokines, most notably TNFα and IL-1 (Sizemore *et al.* 2002, Koul *et al.* 2001, Wang and Baldwin 1996). The transcriptional potential of NF-κB may be regulated at a number of stages (*e.g.* by phosphorylation of IκB, IKK or the

p65/p50 subunits). The kinase CK2 and downstream kinases of PI3K (discussed further in Sections 1.10 and 1.9 respectively) have been shown to phosphorylate components of the NF-κB signalling pathway. For instance CK2 can phosphorylate and induce the degradation of IκB (Kato *et al.* 2003, Meggio and Pinna 2003). The activation of protein kinase B (PKB) by PI3K leads to the phosphorylation of p65 in a pathway that is dependent on IKK-α and -β activity but not the degradation of IκB. PKB has been shown to associate with IKK-α in response to IL-1 and may phosphorylate the kinase directly (Sizemore *et al.* 1999, Madrid *et al.* 2000, Koul *et al.* 2001, Sizemore *et al.* 2002).

Hwang *et al.* (2004) have demonstrated PI3K-dependent stimulation of NF-κB DNA binding and expression from a reporter construct regulated by NF-κB, in response to IFN-γ. This study demonstrated that the induction of iNOS expression by IFN-γ occurred in a PI3K-dependent pathway and that PI3K also regulated the phosphorylation of STAT1 in microglial cells. NF-κB may then function in synergy with, or independent of, STAT1 in the IFN-γ-mediated regulation of iNOS expression (Hwang *et al.* 2004). A study by Sizemore *et al.* (2004) indicated that the expression of a subset of IFN-γ-stimulated genes in MEFs is dependent on the presence of functional IKK but without a requirement for NF-κB. In this case IFN-γ has no effect on the activity of a NF-κB reporter construct (Sizemore *et al.* 2004). NF-κB binding sites have been found alongside GAS/ISRE sites in the promoter of several genes for which the transcription factor may function in synergy with STATs (Hiroi and Ohmori 2003, Liu *et al.* 2001, Ohmori and Hamilton 1995, Ohmori and Hamilton 1993). Alternatively Deb *et al.* (2001) have suggested that PKR (also linked to the activation of STAT1 in response to IFN-γ (Section 1.7)) may activate NF-κB through the phosphorylation of IκB in a JAK1-mediated, but STAT1-independent mechanism (Deb et al 2001, Kumar *et al.* 1997, Yang *et al.* 1995).

1.8.2.1.2 AP-1

The AP-1 family of transcription factors are characterised by a highly conserved basic-leucine zipper domain at the C-terminus. AP-1 transcription factors are homo- or heterodimers composed of members of the Fos (c-Fos, FosB, Fra1, Fra2) and Jun (c-Jun, JunB, JunD) protein families. Signalling is mediated through binding to an

AP-1 site, also known as a TRE (consensus sequence: TGA[C/G]TCA), in the promoter of target genes. They are often involved in constitutive gene expression but are also regulated transcriptionally and by post-translational modifications in response to a number of cytokines and growth factors (*e.g.* IL-1β, TNF-α, EGF, PDGF) (Granger *et al.* 2000, Karin *et al.* 1997, Angel and Karin 1991). AP-1 transcription factors are commonly regulated by MAPKs including ERKs and c-Jun amino-terminal protein kinase (JNK) (Karin 1995) but other kinases also regulate transcriptional activity including CK2 (Lin *et al.* 1992) and downstream effectors of PI3K independent of JNK or the MAPKs, MEK and p38 (Bian *et al.* 2004).

AP-1 transcription factors regulate the expression of many inflammatory genes and often function in synergy with NF-κB (Allport *et al.* 2000, Ahmad *et al.* 1998, Martin *et al.* 1997). Regulation of gene expression by AP-1 plays an important role in the pathogenesis of glomerular inflammatory disease (Granger *et al.* 2000, Sakurai and Sugita 1998) and it is possible that it may also be involved in other inflammatory disease processes, including atherosclerosis.

The promoter of the interferon-inducible HIN-200 family gene IFI 16 has been found to contain a functional binding site for AP-1 transcription factors, in a region critical for constitutive expression of the gene. DNA binding for AP-1 was increased by IFN-γ treatment as was the induction of AP-1 driven reporter genes. This activation of AP-1 appeared to be a primary effect of IFN-γ treatment as increased binding activity was observed after only 10 minutes. Activation of AP-1 binding has therefore been proposed as a STAT-independent mechanism for expression of IFN-γ inducible genes. Other signalling molecules involved in this pathway are unknown and again there may be a requirement for JAKs (Clarke *et al.* 2003).

1.8.2.1.3 C/EBPs

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that contain a conserved basic-leucine zipper (bZIP) domain at the C-terminus. There are six C/EBP isoforms (C/EBP-α, -β, -γ, -δ, -ε, and -ζ) all of which mediate transcription through an optimal C/EBP binding element (consensus sequence: [A/G]TTGCG[C/T]AA[C/T]). C/EBPs have important roles in the regulation of cellular differentiation and proliferation, metabolism and inflammation. The gene

expression of C/EBP family members is regulated by a number of inflammatory mediators including LPS, IL-1, IL-6, IFN-γ and TNF-α. The C/EBP-β isoform is the most highly regulated by posttranslational modifications and its activity is controlled by two central regulatory domains (reviewed in Ramji and Foka 2002, Poli *et al.* 1998). The cytokines IL-6 (Poli *et al.* 1990), TGF-α (Buck *et al.* 1999) and TNF-α (Yin *et al.* 1996), as well as IFN-γ (see below), have been shown to alter the transcriptional activity of C/EBP-β. Kinases that have been shown to phosphorylate C/EBP-β include protein kinase C (PKC) (Trautwein *et al.* 1993) and protein kinase A (PKA) (Trautwein *et al.* 1994).

IFN-γ has been shown to enhance both the DNA binding activity and expression of C/EBP-β (Roy *et al.* 2005, Esteban *et al.* 2004, Salmenpera *et al.* 2003, Xiao *et al.* 2001, Roy *et al.* 2000). C/EBP-β expression has also been shown to be induced by IFN-γ in STAT1-null fibroblasts (Ramana *et al.* 2001). ERK1 and ERK2, activated by IFN-γ through Pyk2, have been described in relation to a novel IFN-γ activated pathway that appears to be independent of JAK1 activation. C/EBP-β is activated through ERKs following stimulation by IFN-γ and induces gene expression via binding to a novel promoter element, GATE, characterised in the IRF9 (p48) promoter (Roy 2002, Hu *et al.* 2001, Xiao *et al.* 2001, Roy *et al.* 2000). This pathway appears to be dependent on STAT1 as neither the activation of ERKs or GATE-binding is induced in STAT1-deficient cells. However, the response is unaffected by deficiency of JAK1 (Hu *et al.* 2001). A separate study has also shown that the activation of C/EBP-β is not attenuated by inhibitors of tyrosine phosphorylation and may therefore be independent of JAK1/JAK2 signalling. In this study, C/EBP-β DNA binding activity was found to be induced by IFN-γ prior to an increase in gene expression (Salmanpera *et al.* 2003). The activity of C/EBP-β has also been shown to be regulated by IFN-γ through mixed lineage kinases (MLK), independent of ERK activation (Roy *et al.* 2005). C/EBP-β may regulate the IFN-γ-stimulated expression of IRF-9 in synergy with the transactivating factor GATE-binding factor (GBF)-1, the expresson of which is induced by IFN-γ (Meng *et al.* 2005, Hu *et al.* 2002). C/EBPs may also operate in synergy with STATs as a complex of IRF and C/EBP has been found to be induced by IFN-γ in the regulation

of IL-18BP gene expression (Hurgin *et al.* 2002). Stat3 and C/EBP-α have been shown to cooperatively regulate gene expression in response to GM-CSF (Numata *et al.* 2005).

1.8.2.1.4 Sp1/Sp3

The Sp family of transcription factors are characterised by three zinc finger motifs at the C-terminus. There are four Sp proteins (Sp1-4) and several closely related transcription factors including BTEB1, TIEG1, TIEG2 and the Kruppel-like factors (XKLFs). Sp1, Sp3 and Sp4 are the most closely related proteins and of these Sp1 and Sp3 are ubiquitously expressed while the expression of Sp4 appears to be confined to specific cell types, particularly in the brain (reviewed in Philipsen and Suske 1999, Suske 1999, Lania *et al.* 1997). Sp1 acts predominantly as a transcriptional activator while Sp3 may act as a repressor or as a transcriptional activator in the context of the promoter (Ross *et al.* 2002, Noti 1997, Dennig *et al.* 1995, Hagen et al. 1994). Sp transcription factors regulate gene expression through binding to a GC-box element (consensus sequence: CACCC) in the promoter of target genes. Sp1 has been found to be important for the constitutive expression of a number of housekeeping genes and those involved in cellular processes such as cell growth, proliferation and apoptosis (Black *et al.* 2001, Black *et al.* 1999), but inducible regulation has also been demonstrated: for example in response to EGF (Merchant *et al.* 1995); TNF-α (Ryuto *et al.* 1996, Giraudo *et al.* 1998); PDGF (Finkenzeller *et al.* 1997); IL-1-β (Tanaka *et al.* 2000); and IL-2 (Too 1997). Constitutive gene expression controlled by GC elements is often downregulated in response to stimuli by the phosphorylation of Sp1/Sp3, so reducing their DNA binding activity (*e.g.* Meissner *et al.* 2004, Ye and Liu 2002, Zhang and Kim 1997). Sp1/Sp3 have been shown to be phosphorylated by kinases including PKA (Rohlff *et al.* 1997), PKB (Pore *et al.* 2004) and CK2 (Armstrong *et al.* 1997, Zhang and Kim 1997)

The GC box is often located near binding sites for other transcription factors suggesting that Sp proteins may act to regulate gene expression in conjunction with other proteins such as NF-κB (Neish *et al.* 1995, Sanceau *et al.* 1995, Perkins *et al.* 1993), Smads (Pardali *et al.* 2000), and C/EBPs (Lee *et al.* 1997), as well as STATs

(Yang *et al.* 2005, Ward and Samuel 2003, Cantwell *et al.* 1998, Too 1997, Look *et al.* 1995, Sanceau *et al.* 1995)

Studies in our laboratory have shown the suppression of macrophage LPL expression by IFN-γ (Tengku-Muhammed *et al.* 1999a, Tengku-Muhammed *et al.* 1998, Tengku-Muhammed *et al.* 1996). Promoter analysis and electrophoretic mobility shift assays (EMSA) revealed that the IFN-γ response was mediated through a reduction in the binding of Sp1 and Sp3 to specific regulatory sequences in the gene promoter. IFN-γ treatment reduced protein levels of Sp3 while the DNA-binding activity of Sp1 was inhibited without a change in protein level. CK2 was found to be responsible for these changes through the phosphorylation of Sp1 and Sp3 (Hughes *et al.* 2002).

1.8.2.3.5 CREB

Transcriptional regulation coupled to cAMP-dependent signalling pathways is mediated through a family of DNA binding proteins comprising the cAMP response element binding protein (CREB), cAMP response element modulator protein (CREM), and activating transcription factors (ATF). These proteins belong to the bZIP class of transcription factors and bind to cAMP-responsive elements (CRE) (consensus sequence: TGACGTCA) in the promoter and enhancer regions of target genes (Antoni 2000).

The activity of CREB has been shown to be both positively and negatively regulated by cytokines (including IFN-γ (Liu *et al.* 2004, Mead *et al.* 2003)) and growth factors (Zhang *et al.* 2004, Jambal *et al.* 2003, Shaywitz and Greenburg 1999). Several kinases are able to phosphorylate CREB including PKA, PKC, CK2 (Meggio and Pinna 2003, Xie and Rothstein 1995, Gonzalez *et al.* 1989) and potentially PKB (Pugazhenthi *et al.* 2000, Du and Montminy 1998). In a previous study, our laboratory demonstrated that the phosphorylation of CREB at Ser-133 was increased by IFN-γ and that this response was inhibited by the CK2 inhibitor apigenin. The activation of CREB was coupled to the upregulation of the inducible cAMP early repressor (ICER) gene expression, mediated by IFN-γ through CRE-like elements known to bind CREB. This reponse was not inhibited by the JAK2 inhibitor AG490 and is potentially JAK-STAT independent (Mead *et al.* 2003) (Section 1.11).

1.8.2.2 Functional redundancy in JAK-STAT signalling

The possibility remains that IFN-γ responsive signalling in STAT1- and JAK1/2-deficient cells is mediated, in the absence of these factors, by other family members. Ramana *et al.* (2005) have recently shown that the regulation of SOCS-3 in STAT1-null BMMs occurs through STAT3 binding to the GAS element. Kotenko *et al.* (1996) have demonstrated that other JAKs can function in place of JAK2 when recruited to the IFN-γR complex. Chimeric receptors were generated in which the extracellular domain of human IFN-γR2 was fused to the intracellular domain of other receptors. Hamster cells expressing the chimeric receptor and human IFN-γR1 were able to support STAT1 dimerisation in response to human IFN-γ (Kotenko *et al.* 1996).

Studies in our laboratory have revealed potential roles for the kinases PI3K and CK2 in the regulation of the genes LPL and inducible cAMP early repressor (ICER) by IFN-γ (Tengku-Muhammad *et al.* 1999b, Hughes et al. 2002, Mead et al. 2003). Additionally, the findings from a review of the current literature, suggest that these kinases may be important mediators in IFN-γ signalling. The potential roles of PI3K and CK2 in the regulation of gene expression by IFN-γ are presented in more detail in the following sections (Section 1.9 and 1.10 respectively).

1.9 ROLE OF PHOSPHOINOSITIDE 3-KINASE IN IFN-γ SIGNALLING

1.9.1 Biological and pathological roles of PI3K-dependent signalling

PI3K is a member of a family of lipid kinases involved in multiple cellular processes including cell growth, proliferation, differentiation, motility, platelet function and cytoskeletal remodelling (Krasilnikov 2000, Ramey and Cantley 1999, Fruman *et al.* 1998). PI3K-dependent signalling is implicated in a number of disease processes: mutations resulting in activation of PI3K signalling pathways lead to tumor formation (Stokoe 2005, Brader and Eccles 2004, Fruman 2004a, Xu *et al.* 2004, Vivanco and Sawyers 2002, Philp *et al.* 2001); while diabetes can be caused by defects in PI3K signalling (Shepherd *et al.* 1998, Cho *et al.* 2001).

In the immune response PI3K may be activated by a variety of stimuli including cytokines (e.g. IL-2, IL-3, IL-4, IL-5), chemokines (e.g. MCP-1, IL-8) and growth

factors (*e.g.* PDGF, EGF) (Fruman 2004b, Fruman and Cantley 2002, Deane and Fruman 2004). Roles for PI3K have been proposed in pulmonary inflammation and asthma (Condliffe *et al.* 2000), and in macrophage accumulation in the inflammatory response (Navarro *et al.* 2003, Gerszten *et al.* 2001, Hirsch *et al.* 2000). A proatherogenic role for PI3K is indicated by the finding that LDL-R-deficient mice receiving bone marrow transplantation with p100δ knock-out bone marrow display reduced atherosclerosis formation in comparison to those receiving wild type bone marrow (Pinderski *et al.* 2005). This is potentially due to an increased accumulation of macrophage foam cells mediated by the proinflammatory effects of PI3K-dependent signalling. Additionally, proliferation and migration of VSMCs in pulmonary tissue has been demonstrated to require PI3K activation (Goncharova *et al.* 2002). OxLDL has also been shown to increase the activity of PKB in these cells and may contribute to the accumulation of SMCs in the atherosclerotic lesion (Chien *et al.* 2003). Recent reviews discuss the function of PI3K in cardiovascular disease (Alloatti *et al.* 2004, Oudit *et al.* 2004).

1.9.2 Isoforms of PI3K

There are three classes of PI3K in mammalian cells (referred to as Class I, II and III). Class I PI3Ks are the most extensively studied and are generally activated in response to extracellular stimuli. Class IA PI3K are composed of a p110 catalytic subunit (α,β and δ) and a regulatory subunit (p85α, p85β, p85γ and p55, p50 that are generated from alternative splicing of the p85α transcript) while Class IB PI3K (PI3Kγ) consists of a p110γ catalytic subunit and a p101 regulatory subunit. There are three Class II isoforms (PI3K-C2α, β and γ), all of which are monomeric enzymes that preferentially phosphorylate phosphoinositol (PI) and PI(4)P, the functions of which are unclear at present. Class III PI3K are heterodimeric, composed of a Vps34P catalytic subunit and a p150 regulatory subunit. They utilise only PI as a substrate and are responsible mostly for the regulation of intracellular vesicular trafficking processes (Hawkins *et al.* 2004, Cantrell 2001, Rameh and Cantley 1999, Fruman *et al.* 1998).

1.9.3 Activation and enzymatic activity of PI3K

In cells of the immune system the activity of PI3K is regulated by a variety of cytokine receptors. Activation of Class IA PI3K occurs in response to a range of cytokines and growth factors through phosphorylation by receptor-associated tyrosine kinases (*e.g.* IL2 and GM-CSF receptors) or receptors with intrinsic tyrosine kinase activity (*e.g.* PDGF, insulin and EGF receptors) (Deane *et al.* 2004, Fruman *et al.* 2004, Fruman and Cantley 2002). PI3Kγ is activated primarily through G-protein coupled receptors often in response to stimulation by chemokines (Curnock *et al.* 2002, Hirsch *et al.* 2000).

Active PI3K catalyzes the formation of 3-phosphorylated phosphatidylinositols (by phosphorylation of the inositol ring of PI) that then serves to activate downstream signalling targets. The most common reaction is the conversion of PI(4,5)P2 to PI(3,4,5)P3 by Class I PI3Ks. Downstream protein kinases are subsequently recruited to the membrane through the binding of pleckstrin homology (PH) domains to PI(3,4,5)P3, where they are brought into proximity with their targets (Hawkins *et al.* 2004, Neri *et al.* 2002, Cantrell *et al.* 2001). The most widely studied of these are 3'-phosphoinositide-dependent protein kinase (PDK)-1 and its substrate PKB (often referred to as Akt), which regulates the activity of a number of downstream effectors including GSK-3, mTOR, IκB and the transcription factor forkhead (FRKD). PDK-1 also activates the kinases p70(S6K) and isoforms of PKC (Bjornsti and Houghton 2004, Yang *et al.* 2004, Cantley *et al.* 2002, Vanhaesenbroeck and Alessi 2000). Negative regulation of PI3K signalling is mediated largely by the protein phosphatases PTEN and SHIP (Hawkins *et al.* 2004, Neri *et al.* 2002, Cantrell *et al.* 2001). These signalling mechanisms are summarised in Figure 1.3. A less well-characterised aspect of PI3K is its function as a protein kinase. For example class I PI3K can phosphorylate serine residues of protein substrates such as insulin receptor substrate (IRS)-1 in response to insulin signalling (Foukas and Shepherd 2004, Lam *et al.* 1994).

1.9.4 PI3K and the JAK-STAT pathway

Several studies have linked PI3K signalling to the JAK-STAT pathway. Stimulation of cells with IFN-γ has been shown to cause the activation of PI3K and increased phosphorylation and activation of its downstream effector PKB (Hwang *et al.* 2004,

Navarro 2003, Nguyen *et al.* 2001). Studies have indicated that, in certain cell lines, the phosphorylation of STAT1 at Serine 727 in response to IFN-γ is dependent on the activation of PI3K and downstream effectors such as PKB (Choudhury *et al.* 2004, Kristof *et al.* 2003, Nguyen *et al.* 2001). While most of the work carried out suggests that PI3K is not necessary for the IFN-γ-stimulated phosphorylation of STAT1 at Tyrosine 701, Hwang *et al.* (2004) have found inhibition of phosphorylation at this site by the specific PI3K inhibitor LY294002 in microglial cells. Other work has suggested a negative role for PI3K in the regulation of STAT1 transcriptional activity, whereby PI3K signals inhibit tyrosine phosphorylation of JAKs or STATs (Krasilnikov *et al.* 2003). JAK1 and JAK2 have both been found to be associated with the p85 regulatory subunit of PI3K in response to stimulation by LPS, leukaemia inhibitory factor (LIF), growth hormone (GH) and prolactin (PRL) (Okugawa *et al.* 2003, Oh *et al.* 1998, Yamauchi *et al.* 1998). IFN-αR also associates with PI3K following stimulation with IFN-α leading to activation of the kinase (Thyrell *et al.* 2004, Uddin *et al.* 2000, Pfeffer *et al.* 1997). Recruitment of PI3K to the IFN-γR complex upon treatment with IFN-γ may lead to its activation through JAKs (Rane and Reddy 2000, Aringer *et al.* 1999). However Nguyen *et al.* (2001) have found that the activation of PI3K in response to IFN-γ is not attenuated in JAK1- or JAK2-deficient cells. It is therefore possible that, in addition to its role in the regulation of STAT1, it may also take part in JAK-STAT-independent signalling pathways through the phosphorylation of other mediators.

A potential PI3K-dependent, STAT1-independent pathway has been proposed by Navarro *et al.* (2003) in the IFN-γ-mediated adhesion of monocytes in inflammation. IFN-γ stimulates the adhesion of both wild type and STAT1-deficient macrophages and this is abrogated by inhibitors of PI3K activity. These inhibitors were not found to affect the STAT1-dependent expression of guanylate binding protein (GBP) (Navarro *et al.* 2003). The adapter molecule c-Cbl, discussed previously in relation to STAT1-independent mediation of IFN-responses (Section 1.8), is also recruited to the membrane in response to PI3K activation (Beckwith *et al.* 1996). Activation of PI3K has been shown to lead to the regulation of a number of transcription factors that modulate inflammatory genes, including NFκB and AP-1 (see Section 1.8).

These factors could potentially be involved in STAT1-independent gene expression in response to IFN-γ or function in synergy with STAT1.

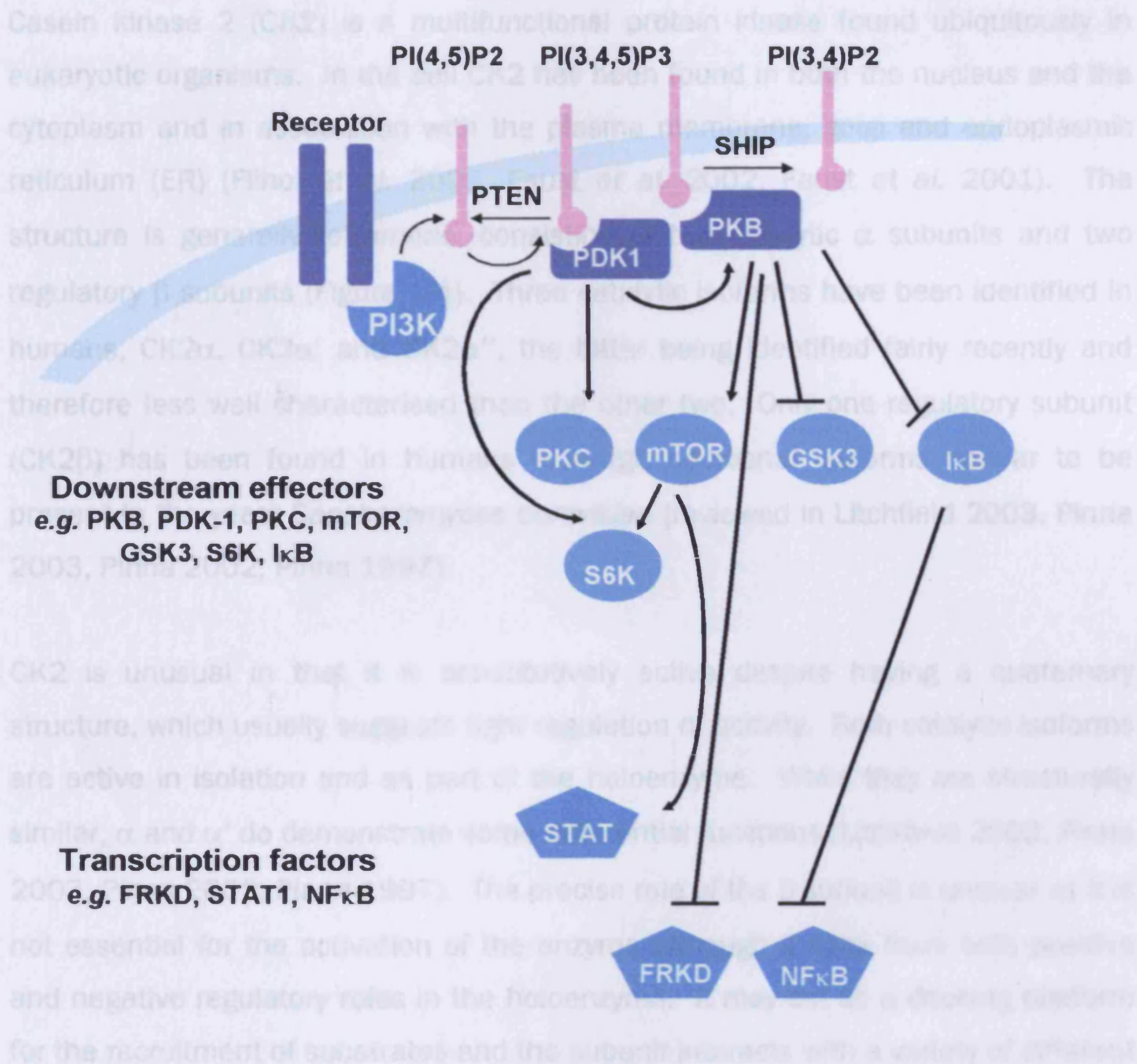


Figure 1.3 Downstream effectors of PI3K

The conversion of PIP2 to PIP3 is catalysed by PI3K, providing a docking site for downstream kinases such as PDK-1 and PKB. PTEN and SHIP antagonise PI3K signalling by converting PIP3 to PI(4,5)P2 and PI(3,4)P2 respectively. PKB is activated by phosphorylation by PDK-1 and these kinases regulate a number of other signalling molecules including PKCs, GSK-3 and mTOR (which subsequently activates S6K). This leads, either directly or indirectly, to the regulation of transcription factors and gene expression.

Abbreviations: FRKD, forkhead; GSK3, glycogen synthase kinase 3; IκB, inhibitor of κB; mTOR, mammalian target of rapamycin; NFκB, nuclear factor κB; PDK1, 3'-phosphoinositide-dependent protein kinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidyl inositol bisphosphate; PIP3, phosphatidyl inositol triphosphate; PKB, protein kinase B; PKC, protein kinase C; PTEN, phosphatase and tensin homologue on chromosome 10; S6K, S6 kinase (p70); SHIP, src homology 2-containing inositol phosphatase; STAT, signal transducer and activator of transcripton.

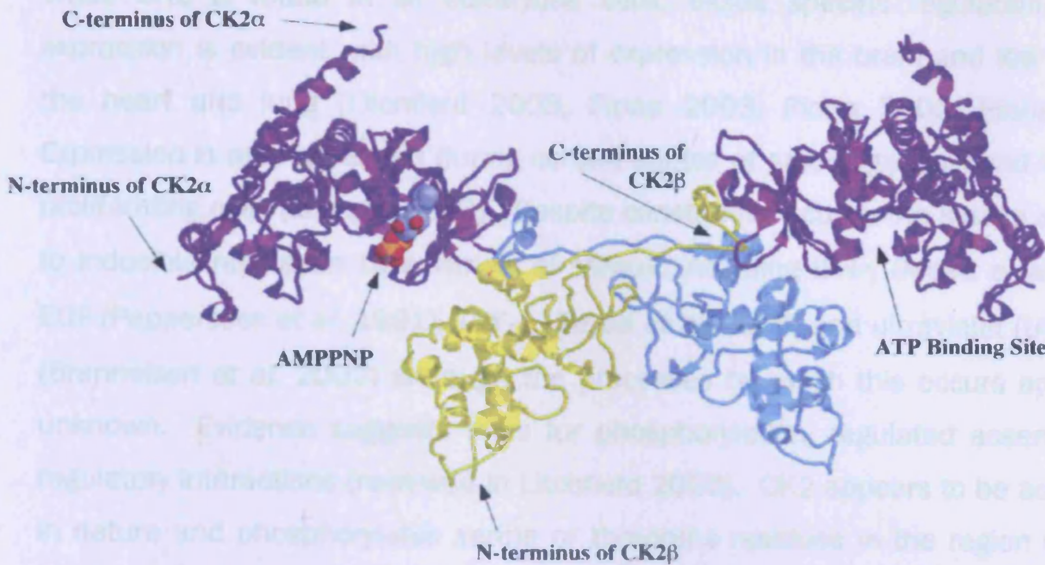
1.10 ROLE OF CASEIN KINASE 2 IN IFN-γ SIGNALLING

1.10.1 Structure and regulation of CK2

Casein kinase 2 (CK2) is a multifunctional protein kinase found ubiquitously in eukaryotic organisms. In the cell CK2 has been found in both the nucleus and the cytoplasm and in association with the plasma membrane, golgi and endoplasmic reticulum (ER) (Filhol *et al.* 2003, Faust *et al.* 2002, Faust *et al.* 2001). The structure is generally tetrameric, consisting of two catalytic α subunits and two regulatory β subunits (Figure 1.4). Three catalytic isoforms have been identified in humans, CK2α, CK2α' and CK2α'', the latter being identified fairly recently and therefore less well characterised than the other two. Only one regulatory subunit (CK2β) has been found in humans although additional isoforms appear to be present in the yeast *Saccharomyces cerevisiae* (reviewed in Litchfield 2003, Pinna 2003, Pinna 2002, Pinna 1997).

CK2 is unusual in that it is constitutively active despite having a quaternary structure, which usually suggests tight regulation of activity. Both catalytic isoforms are active in isolation and as part of the holoenzyme. While they are structurally similar, α and α' do demonstrate some differential functions (Litchfield 2003, Pinna 2003, Pinna 2002, Pinna 1997). The precise role of the β subunit is unclear as it is not essential for the activation of the enzyme although it does have both positive and negative regulatory roles in the holoenzyme. It may act as a docking platform for the recruitment of substrates and the subunit interacts with a variety of different proteins. CK2β has also been suggested to have functions independent of CK2α subunits and is found in cellular locations where CK2α is not present (Bibby and Litchfield 2005). The ability of CK2 to phosphorylate its substrates is dependent upon either ATP or, unusually, GTP binding to the α subunit. Crystallographic studies have shown that the ATP binding pocket of only one of the α subunits of the holoenzyme is occupied at any one time but the physiological relevance of this is not known (Litchfield 2003, Pinna 2003, Pinna 2002, Pinna 1997).

A.



B.

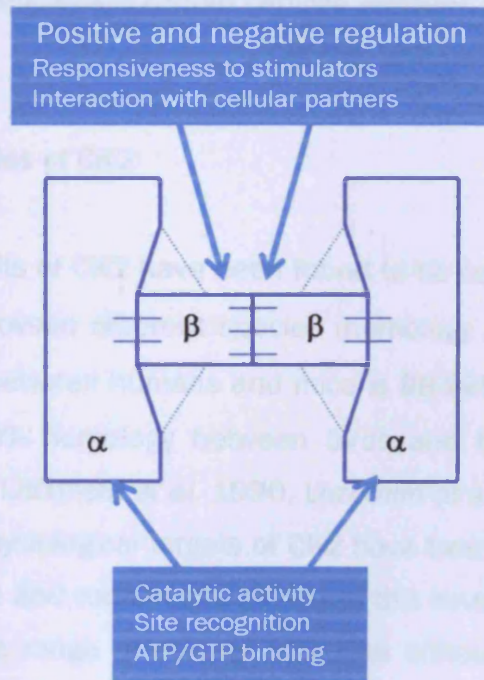


Figure 1.4 Structure of the CK2 tetramer

A. CK2 has a tetrameric structure composed of two catalytic α-subunits (magenta) and two regulatory β-subunits (yellow and blue). The non hydrolysable ATP analogue adenosine 5'-[β,γ-imido]triphosphate (AMPPNP) is present in the ATP binding site of only one of the catalytic CK2α subunits within the CK2 tetramer. The significance of this observation is unknown (adapted from Litchfield 2003).
B. Schematic representation of the CK2 holoenzyme detailing the functional sites (adapted from Pinna 1997)

Abbreviations: ATP, adenosine triphosphate; CK2, casein kinase 2; GTP, guanidine triphosphate.

While CK2 is found in all eukaryotic cells, tissue specific regulation of CK2 expression is evident, with high levels of expression in the brain and low levels in the heart and lung (Litchfield 2003, Pinna 2003, Pinna 2002, Pinna 1997). Expression is also increased during certain stages of embryogenesis and in rapidly proliferating cells (Issinger 1993). Despite constitutive activity the kinase is subject to inducible regulation by a variety of stimuli, including IFN-γ (Mead *et al.* 2003), EGF (Peppercock *et al.* 1991), TNF-α (Sayed *et al.* 2000) and ultraviolet (UV) B light (Brenneisen *et al.* 2002) although the processes by which this occurs are largely unknown. Evidence suggests roles for phosphorylation, regulated assembly and regulatory interactions (reviewed in Litchfield 2003). CK2 appears to be acidophilic in nature and phosphorylates serine or threonine residues in the region of acidic amino acids (in particular asparate and glutamate) although studies have shown that it may also phosphorylate certain tyrosine residues (Wilson *et al.* 1997, Meggio *et al.* 1994).

1.10.2 Biological Roles of CK2

Both α and β subunits of CK2 have been found to be essential for viability and are highly conserved between different species (homology of CK2α between humans and plants is 73%, between humans and mice is 98-99%, CK2β is particularly well conserved with 100% homology between birds and humans) (Xu *et al.* 1998, Maridor *et al.* 1991, Litchfield *et al.* 1990, Lozeman *et al.* 1990). So far over three hundred potential physiological targets of CK2 have been identified, consistent with the pleiotropic nature and constitutive activity of this kinase. These include proteins involved in a diverse range of cellular functions although a large proportion are involved in mechanisms for gene expression and protein synthesis, including: transcription factors (*e.g.* CREB, CREM, c-Jun, IRF-1 and IRF-2); translational elements (*e.g.* eIF2β); and effectors of DNA/RNA structure (*e.g.* RNA binding protein L5); while only a few are enzymes involved in metabolic pathways (*e.g.* glycogen synthase) (Meggio and Pinna 2003).

CK2 is involved in the regulation of basic transcriptional apparatus and substrates include all three RNA polymerases (Pol I, II and III) and TFIIIB-TBP and CK2 activity has been shown to be necessary for synthesis of certain tRNA and 5S rRNA (Cabrejos *et al.* 2004, Hu *et al.* 2003, Johnston *et al.* 2002). A number of studies have implicated CK2 in cellular processes such as modulation of proliferation, apoptosis, regulation of cell morphology and the cell cycle (Canton and Litchfield 2005, Ahmed *et al.* 2002, Sayed *et al.* 2002, Lebrin *et al.* 2000, Pinna and Meggio 1997, Pepperkok *et al.* 1994).

1.10.3 Role of CK2 in disease

Many viral proteins are also substrates of CK2. The constitutive activity of this enzyme may have lead to its adoption as a method for the phosphorylation of proteins essential to the life cycle of certain viruses (*e.g.* latent membrane protein (LMP)-1 and ZEBRA protein of the Epstein-Barr virus (Chi *et al.* 2002, Kolman *et al.* 1993), IE63 protein of the herpes simplex virus (Wadd *et al.* 1999) and the HIV rev protein (Marin *et al.* 2000)). CK2 is also found in increased levels in certain tumours and has been suggested to impart oncogenic potential on cells through promotion of cell survival and increased proliferation (Romieu-Mourez *et al.* 2002, Landesman-Bollag *et al.* 2001a, Landesman-Bollag *et al.* 2001b, Faust *et al.* 1996, Stalter *et al.* 1994). Inhibition of CK2 with dominant negative constructs has been demonstrated to lead to the apoptosis of cancer cells (Wang *et al.* 2001). Increased proliferation through CK2 signalling may also have a role in lesion progression in atherosclerosis. Yamada *et al.* (2005) have shown through microarray analysis that CK2α expression is upregulated in proliferative glomerular lesions in a rat model of the inflammatory disease glomerulonephritis. They subsequently found that expression of antisense CK2α prevented the progression of renal pathology in the disease (Yamada *et al.* 2005). Similar pathways may have a role in the pathology of atherosclerosis.

1.10.4 CK2 and the JAK-STAT pathway

Few studies have addressed the potential role of CK2 in IFN-γ signalling. Our laboratory has shown that CK2 activity is induced by IFN-γ and that this is not inhibited by the JAK2 inhibitor AG490 (Mead *et al.* 2003, Evans, S., personal communication). Use of the pharmacological inhibitor of CK2, apigenin, implicated CK2 in the regulation of LPL and ICER in response to IFN-γ, through the phosphorylation of Sp1/Sp3 and CREB respectively (see Sections 1.8 and 1.11) (Mead *et al.* 2003, Hughes *et al.* 2002). Other transcription factors may also be regulated by CK2 (*e.g.* NFκB) (see Section 1.8). Higashi *et al.* (2003) have shown that CK2 is involved in the IFN-γ-mediated suppression of α2(I) procollagen gene expression through the regulation of Y-box binding protein (YB)-1 in a potential JAK-STAT-independent pathway. They found that apigenin did not affect the phosphorylation (at Tyrosine 701) or nuclear translocation of STAT1 while inhibiting the effect of IFN-γ on the translocation of YB-1. This response was also unaffected by AG490 while the inhibitor prevented the IFN-γ-mediated phosphorylation and nuclear translocation of STAT1 (Higashi *et al.* 2003).

1.11 INDUCIBLE CAMP EARLY REPRESSOR

IFN-γ has been found to stimulate a dramatic, transient increase in ICER expression in the murine macrophage cell line J774.2. This induction was found to be mediated through the phosphorylation and activation of CREB by CK2 (Mead *et al.* 2003). ICER may be potentially responsible for suppressing the expression of several genes regulated in response to IFN-γ through CREs. The LPL gene for example, the expression of which is down-regulated in response to IFN-γ and also in synergy with cAMP, has promoter sites potentially able to bind ICER (Enerback and Gimble 1993). Another study has demonstrated the ability of IFN-γ to suppress CRE- or bZIP containing AP-1 regulated promoters (Saavedra *et al.* 2001).

1.11.1 Structure of ICER

ICER is a member of a family of DNA-binding proteins that mediate transcriptional regulation in response to cAMP. The action of these proteins is mediated through binding to CREs in the promoter of target genes. While CREB, CREM and ATF up-

regulate the expression of target genes, ICER lacks a *trans*-activation domain and hence inhibits transcription either by competing for DNA-binding or by heterodimerisation with other family members (De Cesare and Sassone-Corsi 2000, Shaywitz and Greenburg 1999, Della-Fazia *et al.* 1997, Molina *et al.* 1993).

The CREM gene contains two promoters, P_1 and an internal promoter, P_2. Isoforms of CREM are produced following activation of the P_1 promoter. The alternative promoter (P_2), containing tandem CRE-like elements known as cAMP-autoregulatory elements (CAREs), regulates the expression of ICER. Thus ICER expression can be regulated by CREB/CREM/ATF and by ICER itself. Four isoforms of ICER (ICER I, ICER Iγ, ICER II and ICER IIγ), that appear to be functionally indistinct, result from alternative splicing of the ICER transcript (illustrated in Figure 1.5) (Shaywitz and Greenburg 1999, De Cesare and Sassone-Corsi 2000, Molina *et al.* 1993).

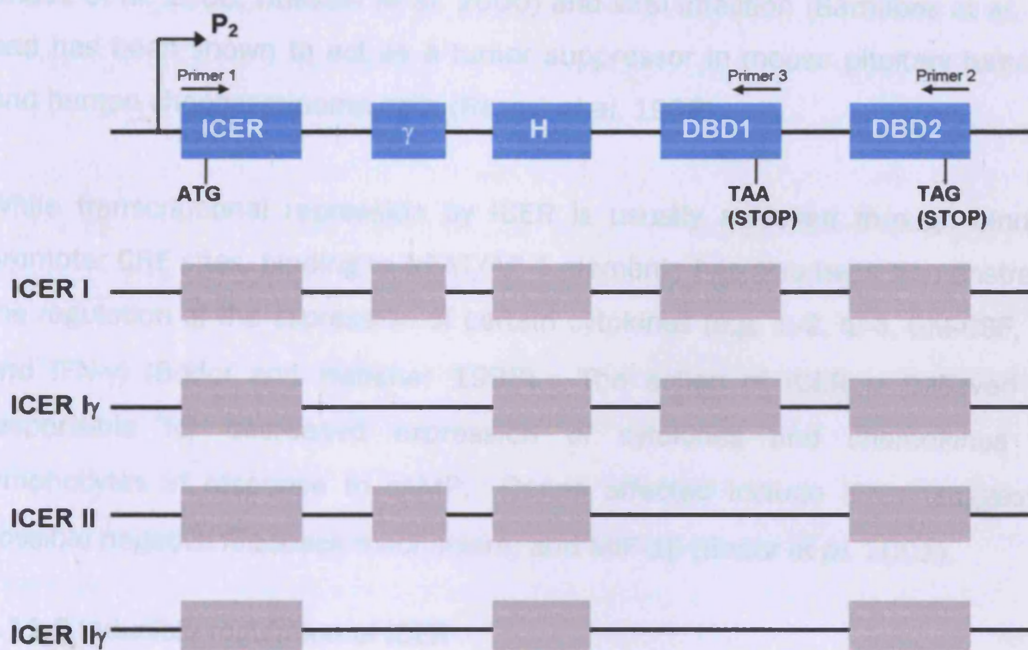


Figure 1.5 Domain structure of the four isoforms of ICER

Boxes represent the different exons (ICER, γ, H, DBD1 and DBD2) of the ICER-encoding region of the CREM gene. Transcription of ICER occurs from promoter P_2. ICER I, ICER Iγ, ICER II and ICER IIγ represent the four mRNA isoforms resulting from alternative mRNA splicing. The ATG initiation codon and stop codons are also indicated. Use of primers 1 and 2 in RT-PCR leads to amplification of mRNA encoding all four ICER isoforms. Primers 1 and 3 are specific for ICER I and ICER Iγ (see Table 2.5).

Abbreviations: DBD, DNA-binding domain; ICER, inducible cAMP early repressor

1.11.2 Biological roles of ICER

The ICER transcript is expressed in a number of cell types including the brain, liver, T cells and macrophages (Mead *et al.* 2003, Mioduszewska *et al.* 2003, Bodor *et al.* 2001, Servillo *et al.* 1997). Negative feedback regulation of ICER means that, in most cases, mRNA expression is transient and allows for cyclic expression of cAMP-responsive genes upregulated by CREB and attenuated by ICER. Rhythmic expression in the pineal gland has implicated ICER in establishing mammalian circadian rhythms through regulation of the hormone melatonin (Link *et al.* 2004, Maronde *et al.* 1999, Foulkes *et al.* 1997). Other roles for ICER include: the regulation of cellular proliferation and the cell cycle; mediation of the stress response; and in the molecular basis of memory (Della Fazia *et al.* 1998, Razavi *et al.* 1998). ICER has also been associated with disease processes such as diabetes (Inada *et al.* 2005, Hussain *et al.* 2000) and viral infection (Barnabas *et al.* 1997) and has been shown to act as a tumor suppressor in mouse pituitary tumor cells and human choriocarcinoma cells (Razavi *et al.* 1998).

While transcriptional repression by ICER is usually achieved through binding to promoter CRE sites, binding to NFAT/AP-1 elements has also been demonstrated in the regulation of the expression of certain cytokines (*e.g.* IL-2, IL-4, GM-CSF, TNF-α and IFN-γ) (Bodor and Habener 1998). The action of ICER is believed to be responsible for decreased expression of cytokines and chemokines in T-lymphocytes in response to cAMP. Genes affected include IFN-γ (suggesting a possible negative feedback mechanism) and MIP-1β (Bodor *et al.* 2001).

1.11.3 Inducible regulation of ICER

ICER is the only inducible transcript of the CREM gene and the expression of ICER has been shown to be stimulated by a range of factors in addition to IFN-γ. These include neuronal growth factor (NGF), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), glucagon, norepinephrine, prostaglandin and neurotransmitters (*e.g.* glutamate) (Inada *et al.* 2005, Hussain *et al.* 2000, Storvik *et al.* 2000, Kameda *et al.* 1999, Uyttersprot *et al.* 1999Mao *et al.* 1998, Monaco *et al.* 1995). While some of these agents act through the cAMP pathway, others mediate their effects via unrelated pathways.

1.12 BIOLOGICAL SIGNIFICANCE OF JAK-STAT-INDEPENDENT PATHWAYS

While the IFN-γ-mediated regulation of a large number of genes appears to be dependent on the JAK-STAT pathway, a growing body of evidence suggests that independent mechanisms may also be involved in the regulation of certain genes. A summary of the potential pathways involved in the regulation of gene expression by IFN-γ is illustrated in Figure 1.6 and the individual components of these pathways are listed in Table 1.9.

There are likely to be certain survival advantages that support the presence of an alternative, JAK-STAT-independent, system for the regulation of gene expression by IFN-γ. For instance, due to the pleiotropic effects of IFN-γ on viral replication, several viruses are known to target IFN-γ signalling largely through inhibition of the JAK-STAT pathway (Hegde *et al.* 2003). Where this occurs, JAK-STAT-independent pathways could provide the host with a mechanism by which key IFN-γ responses can remain active. Studies on STAT1-null mice have revealed that anti-viral responses to certain viruses (Sindbis virus and MCMV) have been higher in these mice than IFN-γR deficient animals, and almost as strong as the wild type response, indicating the utilisation of a STAT1-independent pathway (Gil *et al.* 2001).

STAT1-independent pathways may be involved in stimulating cell growth. Mice lacking STAT1 display increased tumorigenesis, probably due to the increased proliferative response of cells to IFN-γ (Ramana *et al.* 2002). The growth response of human melanoma cells to IFN-γ may also involve conflicting STAT1-dependent (inhibitory) and -independent (stimulatory) signals (Kortylewski *et al.* 2004).

Many STAT1-independently regulated genes have been found to be immunologically important proteins, including chemokines and their receptors (Gil *et al.* 2001). STAT1-independent pathways may therefore play a role in directing cellular migration and inflammation. Recruitment of cells and cellular proliferation within the lesion are important processes in atherogenesis and inhibition of these pathways could be of potential benefit in treating the disease. Conversely STAT1-independent pathways could potentially be linked to atheroprotective processes such as the down-regulation of LPL expression by IFN-γ. It is important now to

identify the signalling molecules and transcription factors involved in JAK-STAT-independent signalling with the aim of identifying potential therapeutic targets for the treatment of disease.

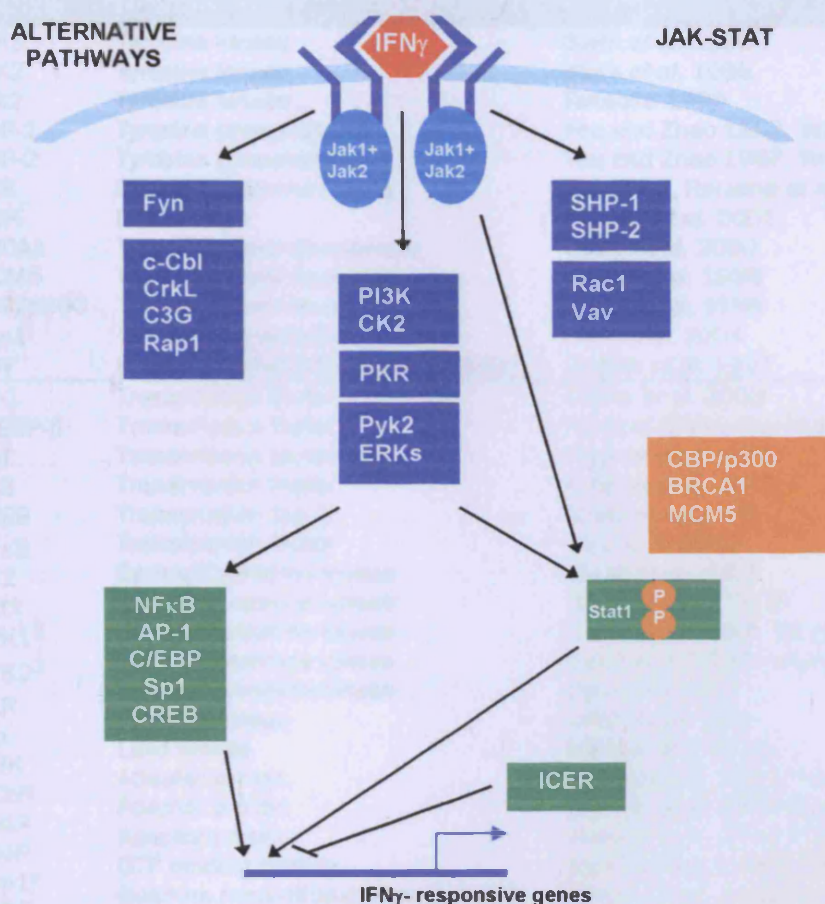


Figure 1.6 Potential signalling pathways involved in modulation of macrophage gene expression by IFN-γ

Binding of IFN-γ to its receptor activates JAKs and mediators such as phosphatases (SHP-1, SHP-2), GTP binding proteins (Rac1, Rap1), adapter proteins (c-Cbl, CrkL), guanine nucleotide exchange factors (Vav, C3G), coactivators (CBP/p300, BRCA1, MCM5), and kinases (CK2, PI3K, PKR, Pyk2, ERKs, Fyn). Some of these mediators are involved in the regulation of JAK-STAT signalling while others potentially regulate the activity of alternative transcription factors such as NFκB, AP-1, C/EBPs, Sp1/Sp3 and CREB. Activation of CREB by CK2, in response to IFN-γ, leads to the upregulation of the transcription repressor ICER that may be responsible for IFN-γ-mediated suppression of gene expression.

Abbreviations: AP-1, activator protein-1; BRCA1, breast cancer 1; C3G, Crk SH3-binding GEF; CBP, CREB-binding protein; c-Cbl, cancer-causing-Casitas-B-lineage lymphoma; C/EBP, CAAT/enhancer binding protein; CK2, casein kinase 2; ERK, extracellular signalling related kinase; Fyn, src/yes-related novel protein tyrosine kinase; ICER, inducible cAMP early repressor; IFN-γ, interferon-γ; JAK, Janus kinase; MCM5, mini-chromosomal maintenance protein 5; NFκB, nuclear factor κB; P, phosphorylated residue; PI3K, phospoinositide 3-kinase; PKR, RNA-dependent protein kinase; Pyk, proline-rich tyrosine kinase; Rac, ras-related C_3-botulinum toxin substrate; Rap, receptor association protein; SHP, Src-homology 2 domain containing protein tyrosine phosphatase; STAT, signal transducer and activator of transcription.

Table 1.9 Proteins involved in IFN-γ signalling pathways

PATHWAY	PROTEIN[1]	PROTEIN TYPE	REFERENCES
JAK-STAT	JAK1	Tyrosine kinase	Stark *et al.* 1998
	JAK2	Tyrosine kinase	Stark *et al.* 1998
	Pyk2	Tyrosine kinase	Takaoka 1999
	SHP-1	Tyrosine phosphatase	You and Zhao 1997, You *et al.* 1999
	SHP-2	Tyrosine phosphatase	You and Zhao 1997, You *et al.* 1999
	PKR	Serine/Threonine kinase	Der 1997, Ramana *et al.* 2000
	PI3K	Lipid kinase	Nguyen *et al.* 2001
	BRCA1	Transcriptional co-activator	Ouchi *et al.* 2000
	MCM5	Transcriptional co-activator	Zhang *et al.* 1998
	CBP/p300	Transcriptional co-activator	Zhang *et al.* 1996
	Rac1	GTP binding protein	Park *et al.* 2004
	Vav	Guanine nucleotide exchange factor	English *et al.* 1997
Potentially STAT-independent	AP-1	Transcription factor	Clarke *et al.* 2003
	C/EBP-β	Transcription factor	Hu *et al.* 2001, Roy *et al.* 2000
	Sp1	Transcription factor	Hughes *et al.* 2002
	Sp3	Transcription factor	Hughes *et al.* 2002
	CREB	Transcription factor	Mead *et al.* 2003
	NFκB	Transcription factor	Deb *et al.* 2001
	CK2	Serine/Threonine kinase	Mead *et al.* 2003
	Raf1	Serine/Threonine kinase	Ramana *et al.* 2000
	ERK1[3]	Serine/Threonine kinase	David *et al.* 1995, Hu *et al.* 2001
	ERK2[3]	Serine/Threonine kinase	David *et al.* 1995, Hu *et al.* 2001
	PKR	Serine/Threonine kinase	Deb *et al.* 2001
	Fyn	Tyrosine kinase	Uddin *et al.* 1997
	PI3K	Lipid kinase	Nguyen *et al.* 2001
	c-Cbl[2]	Adapter protein	Alsayed *et al.* 2000, Platanias 1999
	CrkL[2]	Adapter protein	Alsayed *et al.* 2000, Platanias 1999
	CrkII[2]	Adapter protein	Alsayed *et al.* 2000, Platanias 1999
	Rap1[2]	GTP binding protein	Alsayed *et al.* 2000, Platanias 1999
	C3G[2]	Guanine nucleotide exchange factor	Alsayed *et al.* 2000, Platanias 1999

[1]Proteins may also potentially be involved in both JAK-STAT-independent and -dependent pathways that are uncharacterised at present.
[2]Potential dependence on JAKs is undetermined for the CrkL/C3G pathway (involving c-Cbl, CrkL, CrkII, C3G, Rap1) and for Raf1 and AP-1 activation.
[3]ERK proteins may also be involved in the regulation of STAT1.

Abbreviations: AP-1, activator protein-1; BRCA1, breast cancer 1; CBP, CREB-binding protein; C/EBP-β, CCAAT-enhancer binding protein-β; c-Cbl, cancer-causing-Casitas-B-lineage lymphoma,; C3G, Crk SH3-binding GEF; CK2, casein kinase 2; CREB, CRE-binding protein; ERK, extracellular signalling related kinase; Fyn, src/yes-related novel protein tyrosine kinase; JAK, Janus kinase; MCM5, mini-chromosomal maintenance protein 5; NFκB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; PKR, RNA-dependent protein kinase; Pyk, proline-rich tyrosine kinase; Rac, ras-related C_3-botulinum toxin substrate; Raf, Rous sarcoma associated factor; Rap, receptor association protein; SHP, Src-homology 2 domain containing protein tyrosine phosphatase.

1.13 AIMS OF THE STUDY

Atherosclerosis is a major cause of morbidity and mortality in the Western world. The study of signalling mechanisms involved in the regulation of gene expression, in the progression of the disease, is vital in order to identify new targets for therapeutic intervention. IFN-γ is a key inflammatory mediator in the development of atherosclerosis, and as such, the signalling cascades activated by the cytokine are an important area for scientific research.

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

- Identify novel mediators of the IFN-γ response with respect to the regulation of the expression of a selection of genes
- Analyse the role of the protein kinases PI3K and CK2, implicated by our laboratory in novel regulatory pathways for the expression of two genes (LPL and ICER), in IFN-γ-mediated regulation of gene expression
- Investigate the function of potential JAK-STAT-independent pathways in IFN-γ signalling

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 and dominant negative mutants to determine the role of particular mediators.

Initial investigations led to the identification of a novel gene-specific role for PI3K in the IFN-γ-mediated regulation of the expression of the proatherogenic chemokine MCP-1. The aims were then extended to include:

- Detailed analysis of the pathways involved in the regulation of MCP-1 expression by IFN-γ
- Analysis of factors involved in MCP-1 promoter activation in response to IFN-γ treatment
- Extension of findings regarding the selective function of PI3K in the IFN-γ-mediated regulation of MCP-1 expression to the regulation of other genes associated with the progression of atherosclerosis

Coupled with the continued use of pharmacological inhibitors and dominant negative mutants, these aims were to be achieved by assessing changes in the phosphorylation and activity of downstream effectors in response to IFN-γ treatment, and by analysing promoter binding through EMSA, chromatin immunoprecipitation (ChIP) and the transfection of promoter-reporter constructs. The extension of this work to the regulation of additional genes was achieved using a combination of microarray analysis and RT-PCR.

Following previous studies in which CK2 was implicated in the regulation of ICER expression by IFN-γ, in a mechanism potentially mediated by the CREB/CREM/ATF family of transcription factors, additional aims were to:

- Carry out a detailed analysis of the pathways involved in the regulation of ICER expression by IFN-γ
- Investigate CREs as a potential novel IFN-γ-responsive element

These aims were to be achieved predominantly through transfection-based approaches involving dominant negative mutants and promoter-reporter constructs.

Overall, it was hoped to contribute to the understanding of the signal transduction pathways activated by IFN-γ and leading to alterations in gene expression, particularly with respect to mechanisms that impact on the process of atherogenesis. Such studies will hopefully contribute to the eventual development of novel therapies for the combat of atherosclerosis and CAD.

CHAPTER TWO:

MATERIALS AND METHODS

CHAPTER 2. MATERIALS AND METHODS

2.1 SUPPLIERS

Table 2.1 details chemical reagents and other materials used and the suppliers from which they were purchased.

2.2 PREPARATION OF GLASS AND PLASTICWARE

Glass or plasticware for the isolation and manipulation of DNA, RNA or protein was autoclaved for 20min at 121°C and at a pressure of 975kPa. Solutions were also autoclaved under the same conditions.

2.3 TISSUE CULTURE AND CYTOKINE TREATMENT

2.3.1 Cell lines

The cell lines utilised for the work detailed in this study were: the mouse macrophage cell line, J774.2; the human monocytic cell line, U937; the human hepatoma cell line, Hep3B; and the human endothelial cell line, EA.hy926. J774.2 cells are fully differentiated macrophages with adherent properties. The cell line was re-cloned from the original J774.1 cell line, adapted from the ascites form of a murine reticulum cell sarcoma (J774) (Ralph and Nakoinz 1977, Ralph and Nakoinz 1975, Ralph *et al.* 1975). U937 cells are undifferentiated monocytes grown in suspension, derived from a human histiocytic lymphoma (Ralph *et al.* 1976, Sundstrom and Nilsson 1976). Hep3B cells are fully adherent hepatocytes derived from a human carcinoma (Knowles *et al.* 1980, Aden *et al.* 1979). The EA.hy926 cell line was a generous gift from Dr C S Edgell (University of North Carolina). EA.hy926 cells were derived from human umbilical-vein endothelial cells (HUVECs) fused with the human epithelioma cell line A549 (Rieber *et al.* 1993, Edgell *et al.* 1983).

Table 2.1 Suppliers of chemical reagents and other materials

MATERIALS	SUPPLIER
ChIP-IT™ kit	Active Motif, Rixensart, Belgium
SP600125	Affiniti Research Products, Exeter, UK
SH-6	Alexis Biochemicals, Nottinghamshire, UK
α^{32}P-dCTP ECL Western Blotting Detection Reagent Megaprime DNA Labelling Kit Nick Columns Rainbow Protein Size Markers Random Hexamers (PdN6) X-ray Film	Amersham Biosciences, Buckinghamshire, UK
Acrylamide: Bisacrylamide (29:1) Acrylamide: Bisacrylamide (37.5:1)	Anachem, Luton, UK
DMEM Tissue Culture Medium RPMI 1640 Tissue Culture Medium	Autogen Bioclear, Wiltshire, UK
Endothelial Growth Medium	BD Biosciences, Oxford, UK
Agarose Magnesium Chloride Taq Polymerase (and 10x Buffer)	Bioline, London, UK
AG490 LY294002 Rapamycin	Calbiochem, Nottingham, UK
LB Agar Capsules LB Medium Capsules	DIFCO Laboratories, Surrey, UK
Hep3B Cell Line J774.2 Cell Line U937 Cell Line	European Collection of Animal Cell Cultures (ECACC), Salisbury, UK
EDTA Ethanol Hydrochloric Acid Industrial Methylated Spirits Isopropanol Sodium Chloride Sodium Dodecyl Sulphate Sodium Hydroxide Tris Tri-Sodium Citrate Other General Chemicals	Fisher Scientific, Loughborough, UK
Saran Wrap	Genetic Research Instrumentation, Essex, UK
Ammonium Persulphate Fetal Calf Serum 2-Mercaptoethanol Trypsin/EDTA	Gibco BRL, Paisley, UK
Cell Scrapers Tissue Culture Flasks	Helena Biosciences, Sunderland, UK
High Purity Plasmid Maxi Prep System	Marligen Biosciences, High Wycombe, UK
PVDF membrane	Millipore Ltd., Gloucestershire, UK
DNA Molecular Weight Markers Restriction Endonucleases Anti-PKB Anti-PKB pThr308 Anti-PKB pSer473 Akt Kinase Assay Kit	New England Biolabs, Hertfordshire, UK
Lymphoprep™	Nycomed Pharma, Bridport, UK
Phosphate Buffered Saline Tablets	Oxoid Ltd., Basingstoke, UK
Micro BCA Protein Assay Kit	Pierce, Chester, UK

dNTPs Luciferase Substrate MMLV Reverse Transcriptase (and MMLV 10x Buffer) Passive Lysis Buffer (5x) RNasin™ Wizard SV™ Miniprep Kit	Promega, Southampton, UK
QIAquick™ Gel Extraction Kit Rneasy™ Total RNA Isolation Kit Superfect™ Transfection Reagent	Qiagen, Crawley, UK
Biotinylated-dUTP	Roche, Hertfordshire, UK
Protein A/G Agarose Anti-STAT1 p89/94 Anti-Sp1 X Anti-CK2 α, α' and β Anti-MCP-1	Santa-Cruz Biotechnology Inc., California, USA
Tissue Culture Filters (0.2μm)	Schleicher and Schuell, London, UK
DNA Oligonucleotides	Sigma Genosys, Cambridgeshire, UK
Anti-β-Actin Ampicillin Apigenin Aproptonin Benzamidine Bovine Serum Albumin Bromophenol Blue Ethidium Bromide Ficoll (Type 400) Formaldehyde Formamide Glycerol Leupeptin Mineral Oil Molecular Biology Grade Water PEI Penicillin/Streptomycin PMA PMSF 10x TBE TEMED Tissue Culture Grade Water Tissue Culture Grade DMSO Trypan Blue Solution Tween 20 Wortmannin	Sigma, Poole, UK
Interferon-γ (Mouse) Interferon-γ (Human) SuperArray GEArray™ kit Mouse Atherosclerosis Microarray	Totam Biologicals (Tebu Bio), Cambridgeshire, UK
Anti-STAT1 pSer727 Anti-STAT1 pTyr701 Anti-JAK2 Anti-p85	Upstate Biotechnology, Dundee, UK

2.3.2 Maintenance of cell lines in culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (J774.2, Hep3B, EA.hy926) or Roswell Park Memorial Institute (RPMI) 1640 medium (U937) supplemented with 10% (v/v) heat-inactivated (45min, 56°C) fetal calf serum (HI-FCS) and penicillin (100U/ml)/streptomycin (100U/ml) (pen/strep). HI-FCS and pen/strep were filtered through a 0.2μm sterile filter before addition to the culture medium. Cells were incubated in a humid environment of 5% (v/v) CO_2 at 37°C. Culture medium was replaced every two days.

2.3.3 Subculturing of cell lines

Cells were pelleted by centrifugation (800*g*, 5min), washed once with DMEM (preheated to 37°C) (J774.2, Hep3B, EA.hy926) or RPMI 1640 (U937), and then suspended into fresh medium (10% (v/v) HI-FCS, 1% (v/v) pen/strep) and plated into new flasks at a ratio of 1:4 (*i.e.* cells from one flask were plated into four flasks of the same size). For adherent cells, resuspension was achieved using sterile disposable cell scrapers in the case of J774.2 cells, and by trypsinisation of the extracellular matrix for Hep3B and EA.hy926 cells. Trypsinisation involved the incubation of cells at 37°C (Hep3B, 10min; EA.hy926, 2min) with a volume of trypsin/EDTA adequate to cover the cell monolayer. Trypsin was subsequently inactivated by washing in the presence of FCS.

2.3.4 Human primary monocyte-derived macrophage cell culture

Human blood buffy coats were obtained from the National Blood Service Wales. Buffy coat was underlayered with Lymphoprep™ (Nycomed Pharma) (2:1 (v/v) supernatant:Lymphoprep™) (containing dextran and sodium diatrizoate that cause erythrocyte aggregation) and subjected to centrifugation (800*g*, 20min) to allow sedimination of erythrocytes. The resulting interface was collected and washed with an equal volume of PBS-0.4% (w/v) tri-sodium citrate. Cells were pelleted by centrifugation (800*g*, 5min), resuspended in 0.2% (v/v) saline solution and incubated on ice (30s). An equal volume of 1.6% (v/v) saline solution was added and cells pelleted immediately by centrifugation (800*g*, 5min). The pellet was then washed 6-8 times in PBS-0.4% (w/v) tri-sodium citrate to remove

contaminating platelets. Remaining cells were plated in RPMI 1640 (1% (v/v) pen/strep) and incubated at 37°C, 5% (v/v) CO_2 for 6h in order to allow the adherence of monocytes. After this time, the cells were washed twice with PBS and given fresh medium supplemented with 5% (v/v) human serum. Serum was obtained by centrifugation of clotted human blood (30ml) (1400g, 15min). Culture medium was replaced every 24h for 7-10 days, over which time the cells differentiated from monocytes to macrophages.

2.3.5 Human umbilical vein-endothelial cell culture

HUVECs were extracted from human tissue and kindly donated by Karolina Taylor (Heath Hospital, Cardiff). Confluent cells were obtained at passage 3, in Endothelial Cell Growth Medium (BD Biosciences), and harvested the same day.

2.3.6 Treatment of cells with IFN-γ

All cells were between passage 2 and 9. J774.2 cells were grown to approximately 60% confluence before treatment, Hep3B and EA.hy926 cells to approximately 80% and U937s were plated at a density of 3 x 10^5/ml. Human or mouse IFN-γ, as appropriate, was added to the culture medium at 1000U/ml following a 4h pre-incubation period in fresh culture medium (10% (v/v) HI-FCS) or following transfection procedures (Section 2.4). Treated cells were incubated as before at 37°C, for the appropriate time, after which the relevant extraction procedures were carried out.

2.3.7 Treatment of cells with inhibitors

Inhibitors were added to the cell culture medium for a pre-treatment period of 1h prior to the addition of IFN-γ. Control cells were treated with an appropriate volume of a vehicle control, typically DMSO. Table 2.2 details the pharmacological inhibitors used and their mode of action.

Table 2.2 Action of pharmacological inhibitors used

INHIBITOR[1]	CHEMICAL NAME	FORMULA	TARGET	MECHANISM	REFERENCE
Actinomycin D	8-amino-N-(2-amino-4,6-dimethyl-3-oxo-phenoxazin-1-yl)carbonyl-N'-[8-amino-4,6-dimethyl-7-oxo-9-[[3,6,10-trimethyl-7,14-bis(1-methylethyl)-2,5,8,12,15-pentaoxo-9-oxa-3,6,13,16-tetrazabicyclo[14.3.0]nonadec-11-yl]carbamoyl]phenoxazin-1-yl]carbonyl-4,6-dimethyl-7-oxo-N,N'-bis[3,6,10-trimethyl-7,14-bis(1-methylethyl)-2,5,8,12,15-pentaoxo-9-oxa-3,6,13,16-tetrazabicyclo[14.3.0]nonadec-11-yl]-1,9-bis[[3,6,10-trimethyl-7,14-bis(1-methylethyl)-2,5,8,12,15-pentaoxo-9-oxa-3,6,13,16-tetrazabicyclo[14.3.0]nonadec-11-yl]carbamoyl]phenoxazine-1,9-dicarboxamide	$C_{62}H_{86}N_{12}O_{16}$	RNA polymerase	Reversible competitor of DNA binding	Goldberg *et al.* 1962
AG490	α-Cyano-(3,4-dihydroxy)-N-benzylcinnamide	$C_{17}H_{14}N_2O_3$	JAK2	Reversible competitor of substrate binding	Meydan *et al.* 1996
Apigenin	4',5,7-Trihydroxyflavone	$C_{15}H_{10}O_5$	CK2	Reversible ATP/GTP-competitive inhibitor of CK2	Critchfield *et al*, 1997
LY294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one	$C_{19}H_{17}NO_3$	PI3K	Reversible ATP-competitive inhibitor of PI3K	Vlahos *et al*, 1994
Rapamycin	9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-(2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethyl)-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido(2,1-c)(1,4)oxaazacyclohentricontine-1,5,11,28,29(4H,6H,31H)-pentone	$C_{51}H_{79}NO_{13}$	mTOR	Complex formation with FKBP-12 inhibits mTOR activity	Chen *et al.* 1995
SH-6	D-2,3-Dideoxy-*myo*-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate	$C_{28}H_{57}O_9P$	PKB	Reversible competitor of PH-domain binding	Kozikowski *et al.* 2003
SP600125	1,9-Pyrazoloanthrone	$C_{14}H_8N_2O$	JNK/ SAPK	Reversible ATP-competitive inhibitor of JNK1, -2 and -3	Bennett *et al*, 2001
Wortmannin	KY12420	$C_{23}H_{24}O_8$	PI3K	Irreversible covalent binding to the ATP-binding site of the p110 catalytic subunit	Arcaro and Wymann 1996

[1]Further information on each of these inhibitors can be found on the manufacturer's website.

2.3.8 Trypan blue exclusion assay

Trypan blue solution (0.4% (w/v)) was added to the cell culture medium at a ratio of 1:100 (v/v) and incubated at 37°C (10min). The number of cells taking up the blue stain was estimated for adherent cells by counting those in a representative area. The approximate proportion of dead cells was calculated from this. For suspension cells a haemocytometer was used to create a monolayer and the same procedure was carried out.

2.4 DNA TRANSFECTION OF CELL LINES

All cells were between passage 2 and 7. U937 cells were plated at a density of 7.5 x 10^5/ml in RPMI 1640 (3% (v/v) HI-FCS), 4h prior to transfection. At the time of transfection the differentiating agent phorbol 12-myristate 13-acetate (PMA) (1μM) was also added to these cells. Hep3B cells were grown to approximately 60% confluence and fresh medium (DMEM 10% (v/v) HI-FCS) was added 4h before transfection.

2.4.1 SuperFect™ transfection

The transfection method was carried out as described by the manufacturers (Qiagen). The DNA/SuperFect™ complex was prepared by diluting DNA (1-2μg) to a concentration of 20ng/μl in RPMI 1640. SuperFect™ solution (4μl/μg DNA) was added and the mixture was vortexed and incubated at room temperature (10min). The complex was then suspended in RPMI 1640 (200μl/μg DNA) and added dropwise to the cells. Cells were treated with mediators immediately following transfection.

2.4.2 Polyethylenimine transfection

Plasmid DNA (5-10ug) was diluted to a volume of 10μl in 5% (w/v) glucose. Polyethylenimine (PEI) solution (5.625mg/ml, pH 7.2) (1.5μl) was added to the DNA and the resulting complex immediately suspended in DMEM (10% (v/v) HI-FCS) (1ml) and added dropwise to cells. Cells were incubated at 37°C (24h), washed twice in DMEM and treated with mediators as usual.

2.4.3 Preparation of cell extracts for determination of reporter gene activity

Transfected cells washed once with phosphate buffered saline (PBS), and resuspended in 1x Passive Lysis Buffer (Promega). After vortexing, the lysate was incubated for 10min at room temperature. Cell debris was pelleted by microcentrifugation (11,000rpm, 2min) (in all cases microcentrifugation was carried out using a Heraeus Biofuge Pico) and the supernatant retained and used to assay reporter gene activity.

2.4.4 Luciferase assay

Extracts from transfected cells (60µl) were mixed with a luciferase substrate (Promega) (100µl) and the fluorescent intensity (counts) was determined using a Turner Designs 50/50 Luminometer set at a sensitivity value of 70% with a 2s delay period and 20s integrate period. The recorded number of counts is relative to the amount of luciferase expressed by the reporter plasmid and hence the activity of the promoter sequence of interest. Counts were normalised to the amount of protein (µg/ml) in each lysate, determined as described in Section 2.6.4.

2.4.5 Plasmid constructs used for transfection based assays

Plasmid maps for all expression and reporter vectors can be found in Appendix I. Table 2.3 details the restriction endonuclease digestion used to verify the identity of each plasmid (Section 2.5.11)

2.4.5.1 pCRELuc

The pCRELuc plasmid was purchased from Stratagene and is based on the pLuc-MCS expression vector. Transcription is regulated by a TATA box and four upstream CRE consensus enhancer elements ($(AGCC\underline{TGACGTCA}GAG)_4$).

2.4.5.2 MCP-1Luc

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 is inserted into the pGL2-Basic luciferase reporter vector (Promega) (Zhou *et al.* 1998).

2.4.5.3 pC/EBPx4Luc

The pC/EBPx4Luc plasmid was created by Dr T Hughes (Cardiff University) and is based on the pGL2-Promoter luciferase reporter vector (Promega). Transcription is regulated by a SV40 promoter and four upstream C/EBP consensus enhancer elements.

2.4.5.4 pNFκBLuc

The plasmid pNFκBLuc was obtained from Dr D Leeke (University of Reading) and is based on the luciferase expression vector pTALLuc (Clontech) containing an insert of four NFκB consensus enhancer elements.

2.4.5.5 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.
Further information can be found at:
http://www.rockefeller.edu/labteach/darnell/request.php.

2.4.5.6 JAK1/JAK2 and STAT1

Plasmid constructs for dominant negative JAK1, JAK2 and STAT1, were donated by Prof J E Darnell (Rockefeller University, New York). Dominant negative JAK1 and JAK2 are present as inserts in the pRK5 vector. A three amino acid change (FWYAPE to CTYAPV) in the conserved region VIII of the kinase domain of JAK1 impairs the catalytic function (pRK5-JAK1). Dominant negative JAK2 contains a single amino acid mutation (Lysine to Arginine) at the ATP-binding site, resulting in reduced ATP-binding and catalytic activity (pRK5-JAK2) (Zong *et al.* 2000). Wild type and mutant STAT1 expression plasmids utilise the pRC/CMV vector (Invitrogen). STAT1 constructs include: STAT1-α FLAG, expressing the wild type protein; STAT1 S727A, a partially active mutant with a serine to alanine change at the phosphorylation site Serine 727; STAT1-β FLAG, a truncated form of STAT1-α with reduced function; and STAT1 701F FLAG, a dominant negative mutant containing a tyrosine to phenylalanine change at the JAK kinase phosphorylation site (Tyrosine 701) (Osaki *et al.* 2003).
Further information about these plasmids can be found at:
http://www.rockefeller.edu/labteach/darnell/request.php.

2.4.5.7 CK2

The CK2 dominant negative plasmid construct, pSG-CK2α-K68A, was a generous gift from Drs E M Chamburg and C Cochet (INSERM, Grenoble, France). This construct contains a kinase inactive mutant (lysine to alanine change at residue 68 within the ATP-binding domain) of the CK2α catalytic subunit in the pSG plasmid vector (Stratagene) (Lebrin *et al.* 2001).

2.4.5.8 PKB

The plasmid pcDNA3 HA-PKB AAA was donated by Dr B Hemmings (Basel). An inactive form of PKBα, with a mutation to alanine at residue 179K in the ATP-binding site and residues 308T and 473S that must be phosphorylated in the active kinase, is inserted into the pcDNA3 expression vector (Invitrogen).

2.4.5.9 IκB

The plasmid pcDNA3 IκB was donated by Dr D Krappman (Berlin). Expression of the endogenous super-repressor IκBα (human) from the pcDNA3 expression vector (Invitrogen) acts to inhibit NF-κB signalling in a dominant negative manner (Krappmann *et al.* 1996).

2.4.5.10 LIP

The plasmid pCS2+ LIP was created by Dr S Kousteni (Cardiff University). cDNA encoding *Xenopus* LIP was cloned into the pCS2+ expression vector (Kousteni *et al.* 1998). LIP is an isoform of C/EBP-β that lacks the *trans*-activation domain and thereby inhibits the expression of promoters regulated by C/EBP-β by competing for promoter binding.

2.4.5.11 ICER

The plasmid pcDNA3 ICER was created by Dr J Mead (Cardiff University). cDNA (522bp) encoding murine ICER I was inserted into the pcDNA3 expression vector (Promega).

Table 2.3 Restriction endonuclease digestion of plasmid constructs

PLASMID CONSTRUCT		VECTOR	RESTRICTION ENDONUCLEASE (S)	FRAGMENTS (kb)
pCRE	Luc	pLuc	BamHI	2.7, 3.1
MCP-1	Luc[213]	pGL2-Basic	XhoI, KpnI	5.7, 0.3
C/EBPx4	Luc	pGL2-Promoter	XmaI, XhoI	5.8,
NFκBx4	Luc	pTAL	XbaI, HindIII	3.3, 1.7
3xly6e	Luc	pZluc-TK	SmaI	6.0
JAK1	Dominant negative	pRK5	EcoRI, BamHI	4.7, 1.6, 0.6, 1.2
JAK2	Dominant negative	pRK5	EcoRI, BamHI	4.7, 2.4, 0.6, 0.2
STAT1	α-FLAG	pRC/CMV	Hind III	6.0, 1.7
	β-FLAG	pRC/CMV	Hind III	6.0, 1.7
	Dominant negative (α-S727A)	pRC/CMV	Hind III	6.1, 1.7
	Dominant negative (αY701F FLAG)	pRC/CMV	Hind III	6.0, 1.7
CK2	Dominant negative	pSG5	EcoRI	1.5, 6.0
PKB	Dominant negative	pcDNA3	NheI, KpnI	6.2, 0.4, 0.3
LIP	-	pcS2+	XbaI	4.7
IκB	-	pcDNA3	-	-
ICER I	-	pcDNA3	BamHI, EcoRI	5.4, 0.5

Abbreviations: A, alanine; C/EBP, CCAAT/enhancer binding protein; CK2, casein kinase 2; CRE, cAMP responsive element; IκB, inhibitor of κB; ICER, inducible cAMP early repressor; F, phenylalanine; JAK, janus kinase; Luc, luciferase; MCP-1, monocyte chemoattractant protein-1; NFκB, nuclear factor κB; p, plasmid; PKB, protein kinase B; S, serine; STAT, signal transducer and activator of transcription; Y, tyrosine.

2.5 RNA/DNA RELATED TECHNIQUES

2.5.1 Stock solutions

Table 2.4 Composition of stock solutions used for electrophoresis of DNA/RNA

SOLUTION	COMPOSITION
5x DNA loading dye	0.5x TBE 40% (v/v) glycerol 0.25% (w/v) bromophenol blue

All solutions were prepared with sterile double distilled water (ddH_2O)

2.5.2 RNA isolation

Extraction of RNA from cells was carried out using the RNeasy™ kit according to the instructions supplied by the manufacturer (Qiagen). Where RNA was extracted for use in microarray analysis a DNaseI step was included, carried out as specified in the manufacturer's protocol (Qiagen). The purity and concentration of RNA obtained was calculated by measuring the absorption of light at wavelengths of 260nm and 280nm with a Hitachi U-1800 spectrophotometer. The quality of RNA was also analysed by resolving 1µg on a 1% (w/v) agarose gel (Section 2.5.4).

2.5.3 Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR involves two stages: firstly the synthesis of cDNA from RNA using the enzyme reverse transcriptase; and secondly a standard PCR reaction using this cDNA to obtain a specific product.

2.5.3.1 Synthesis of cDNA (RT)

Total RNA (1µg) was incubated with random hexamers (PdN6) (200pmol) diluted to a volume of 14µl with tissue culture grade water at 70°C (5min). Following this the reactions were placed immediately on ice (5min). Molony murine leukaemia virus (MMLV) reverse transcriptase (200U), MMLV 5x Buffer and dNTPs (1µl, 10mM) were added to give a final reaction volume of 20µl which was incubated at 37°C (1h). Solutions were subsequently diluted at a ratio of 1:5 in tissue culture grade water.

2.5.3.2 PCR

PCR reactions contained 1x Buffer (Bioline), dNTPs (100μM), Taq polymerase (1U) and Forward and Reverse primers (1μM). Concentration of magnesium chloride ($MgCl_2$) and cDNA was varied and for some primer sets DMSO was required. Cycles included a denaturation step of 95°C for 30 sec; primer annealing for 1min at a variable temperature; and an extension period at 72°C for 2min. An initial denaturation step of 95°C for 5min and a final extension at 72°C for 10min was also included. The number of cycles was selected so that final products were generated in the exponential phase of amplification. Primer sequences, $MgCl_2$ concentration, cDNA volume, DMSO requirement, annealing temperature and number of cycles are listed in Table 2.5. Primer sequences were either taken from the sources referenced or generated using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). Temperature cycling was carried out on either a Biometra TRIO Thermoblock (for which reactions were overlayed with mineral oil) or a Peltier Thermal Cycler (PTC-200).

2.5.4 Agarose gel electrophoresis

PCR products were resolved on 1-2% (w/v) agarose gels and the fragment size determined by comparison to standard DNA molecular weight markers (Appendix II). Gels were made using 0.25x TBE and contained ethidium bromide (0.5μg/ml). Electrophoresis was carried out on a Fisherbrand Horizontal Gel Unit at 85V, with 0.25x TBE as running buffer. Samples were mixed with DNA loading dye (1x) before adding to the wells. DNA bands were visualised under UV using a Syngene Gel Documentation System.

Table 2.5 PCR primer sequences and reaction conditions

GENE (REFERENCE)	PRIMER SEQUENCES	PRODUCT SIZE (bp)	cDNA (μL/50μL REACTION)	$MgCl_2$ CONC (mM)	DMSO (% V/V)	ANNEALING TEMPERATURE (°C)	NO. CYCLES
28S rRNA (Kong *et al.* 1999	For: 5'-TGA ACT ATG CTT GGG CAG GG -3' Rev: 5'-AGC GCC ATC CAT TTT CAG GGG -3'	513	5	1	-	57	11
ABCA1 (Klucken *et al.*)	For: 5'-GTT GGA AAG ATT CTC TAT ACA CCT GA -3' Rev: 5'-CGT CAG CAC TCT GAT GAT GGC CTG -3'	690	10	1	-	60	27
hβ-Actin (Sabatakos *et al.* 1998)	For: 5'-ATG ATA TCG CCG CGC TCG-3' Rev: 5'-CGC TCG GTG AGG ATC TTC A-3'	580	10	1.5	-	57	18
mβ-Actin (Sabatakos *et al.* 1998)	For: 5'-TGG AGA AGA GCT ATG AGC TGC CTG-3' Rev: 5'-GTG CCA CCA GAC AGC ACT GTG TTG-3'	204	10	1	5	58	18
mCCL11 (Michalec *et al.* 2002)	For: 5'- GCT CCA CAG CGC TTC TAT TC-3' Rev: 5'-TTG TGG CAT CCT GGA CC -3'-	248	10	1.5	-	60	40
mCCL20 (Shirane *et al.* 2004)	For: 5'-TGC TCT TCC TTG CTT TGG CAT GGG TA -3' Rev: 5'-TCT GTG CAG TGA TGT GCA GGT GAA GC -3'	365	10	1.5	-	60	35
mCCR2 (Fife *et al.* 2001)	For: 5'-GGT CAT GAT CCC TAT GTG G -3' Rev: 5'-CTG GGC ACC TGA TTT AAA GG -3'	253	10	1.5	-	60	30
mCCR2 (Schadde *et al.* 2000)	For: 5'-AAA GGA AAT GGA AGA CAA TAA TAT G-3' Rev: 5'-AAA GGC AAA CTG TCA CTT ACT TTA C-3'	1149	10	1.5	-	56	30
mCD36 (Yoshida *et al.* 1997)	For: 5'-CAG CCC AAT GGA GCC ATC-3' Rev: 5'-CAG CGT AGA TAG ACC TGC-3'	487	10	1.5	-	56	26
mCD44 (Kimura *et al.* 2002)	For: 5'-GTA CAT CAG TCA CAG ACC TAC-3' Rev: 5'-CAC CAT TTC CTG AGA CTT GCT-3'	598	10	1.5	-	56	30
GAPDH (Sabatakos *et al.* 1998)	For: 5'-CCC TTC ATT GAC CTC AAC TAC ATG G-3' Rev: 5'-AGT CTT CTG GGT GGC AGT GAT GG-3'	455	5	2	-	60	18
mICAM1 (Sano *et al.* 2005)	For: 5'-GTC CGC TTC CGC TAC CAT CA-3' Rev: 5'-ACT GCT GTT TGT GCT CTC CT-3'	508	10	1.5	-	60	30
hICER I/II (Peri *et al.* 2001, Peri *et al.* 2002)	For: 5'-CTG ATG AGG AAA CTG AAC TTG-3' Rev: TCG GCT CTC CAG ACA TTT TAC-3'	657 (ICERI) 257 (ICER II)	5	1.5	-	56	30

mICER I (Hussain *et al.* 2000)	For: 5'-CAA AAG CCC AAC ATG GCT G-3' Rev: 5'-CCA ATT CAC ACT CTA CAG CAG-3'	522 (ICER I)	5	1.5	-	65	28
mICER I/II (Storvik *et al.* 2000)	For: 5'-ATG GCT GTA ACT GGA GAT GAA ACT-3' Rev: 5'-CTA ATC TGT TTT GGG AGA GCA AAT GTC-3'	657 (ICER I) 257 (ICER II)	5	1.5	-	65	28
mIL-10 (Moore *et al.* 1990)	For: 5'-TCC TTA ATG CAG GAC TTT AAG GGT TAC TTG-3' Rev: 5'-GAC ACC TTG GTC TTG GAG CTT ATT AAA ATC-3'	257	10	1.5	-	60	38
miNOS (Hwang *et al.* 2004)	For: 5'-CAA GAG TTT GAC CAG AGG ACC-3' Rev: 5'-TGG AAC CAC TCG TAC TTG GGA-3'	656	10	1.5	-	60	28
mIP-10 (Sgadari *et al.* 1996)	For: 5'-ACC ATG AAC CCA AGT GCT GCC GTC -3' Rev: 5'-GCT TCA CTC CAG TTA AGG AGC CCT -3'	312	10	1.5	-	64	26
mITAC (Hamilton *et al.* 2002)	For: 5'-GAA CAG GAA GGT CAC AGC CAT AGC -3' Rev: 5'-ATG AGG CGA GCC TGC TTG GAT CTG-3'	252	10	1.5	-	66	26
mJun-B	For: 5'-GAC GAC CTG CAC AAG ATG AA-3' Rev: 5'-ATG TGG GAG GTA GCT GAT GG-3'	225	10	1.5	-	59	27
mLPL (Irvine *et al.* 2005)	For: 5'-CAT TTA CCA GGG GGT CAC -3' Rev: 5'-AGG CAG AGC CCT TTC TCA-3'	278	10	2	-	60	26
hMCP-1	For: 5'-CTT CTG TGC CTG CTG CTC ATA GCA-3' Rev: as mouse	219	10	1.5	-	65	30 /25[1]
mMCP-1	For: 5'-CTT CTA TGC CTC CTG CTC ATA GCT-3' Rev: 5'-CTT GGG GTC AGC ACA GAT CTC CTT-3'	219	10	1.5	-	65	25
mMig (Sgadari *et al.* 1996)	For: 5'-ACT CAG CTC TGC CAT GAA GTCCGC -3' Rev: 5'-AAA GGC TGC TCT GCC AGG GAA GGC-3'	479	10	1.5	-	66	26
mMIP-1β (Kawakami *et al.* 1999)	For: 5'-GGA ATT CTG CAG TCC CAG CTC TGT GCA A-3' Rev: 5'-GGA ATT CCA CAG TCA TAT CCA CAA TAG-3'	582	10	1.5	-	62	28
mMIP-1α (Sgadari *et al.* 1996)	For: 5'-CTC AAC ATC ATG AAG GTC -3' Rev: 5'-GGC ATT CAG TTC CAG GTC -3'	285	10	1.5	-	57	30
mMMP9 (Chen *et al.* 2005)	For: 5'-GCG CCA CCA CAG CCA ACT ATG-3' Rev: 5'-TGG ATG CCG TCT ATG TCG TCT TTA-3'	379	10	1.5	-	64	30

mNFκB (Sano *et al.* 2005)	For: 5'-AGC AGA CTC CAC TCC ACT TG-3' Rev: 5'-GAC ATC AGC CCC ACA TTT CA-3'	398	10	1.5	-	60	30
mRANTES (Sgadari *et al.* 1996)	For: 5'-GTA CCA TGA AGA TCT CTG CA -3' Rev: 5'-TCT ATC CTA GCT CAT CTC CA -3'	287	10	1.5	-	60	30
mSOCS-1 (Egwuagu *et al.* 2002)	For: 5'-CTC GAG TAG GAT GGT AGC ACG C-3' Rev: 5'-CAT CTT CAC GCT GAG CGC GAA GA-3'	372	5	1.5	5	60	28
mSR-B1 (Srivastava 2003)	For: 5'-TTT CAG CAG GAT CCA TCT GGT GGA-3' Rev: 5'-AGT TCA TGG GGA TCC CAG TGA C-3'	480	10	1.5	-	60	28
mTNF-α (Fox and Danska 1997)	For: 5'-ATG AGC ACA GAA AGC ATG ATC-3' Rev: 5'-CCA AAG TAG ACC TGC CCG GAC-3'	910	10	1.5	-	56	26
pGL2 (Promega)	GL1: 5'-TGT ATC TTA TGG TAC TGT AAC TGA-3' GL2: 5'-AAG AAA TAC AAA AAG CGC AGA AGG T-3'	For empty vector: 135	-	2	-	55	30
hGAPDH-ChIP (Active Motif)	For: 5'-TAC TAG CGG TTT TAC GGG CG -3' Rev: 5'-TGC CAA AGC CTA GGG CAG AG-3'	166	5 (ChIP)	2.5	-	59	40
hMCP-ChIP (-288--95)	For: 5'-CCC ATT TGC TCA TTT GGT CT -3' Rev: 5'-CTT ATT GAA AGC GGG CAG AG -3'	194	5 (ChIP)	1.5	-	59	40
hNegative-ChIP (Active Motif)	For: 5'-ATG GTT GCC ACT GGG GAT CT-3' Rev: 5'-TGC CAA AGC CTA GGG GAA GA-3'	174	5 (ChIP)	2.5	-	59	40

[1]Hepatocytes, 30 cycles; Macrophages/Endothelial cells, 25 cycles

Abbreviations: ChIP, chromatin immunoprecipitation; For, forward primer sequence; h, human; m, mouse; Rev, reverse primer sequence.

2.5.5 Densitometric analysis

The intensity of bands from agarose gels was analysed using GeneTools™ (Syngene) software.

2.5.6 Bacterial strains and vectors

Table 2.6 indicates the genotype of the *Escherichia Coli* (*E. Coli*) strains used in this work. Plasmid vector maps can be found in Appendix I.

Table 2.6 Genotype of *E.Coli* strains used

BACTERIAL STRAIN	GENOTYPE	REFERENCE
DH5-α	*supE44ΔlacU169(ϕ80lacZΔM15)hsd17 recA1endA1gyrA96thi-1recA1*	Hanahan 1983a, Hanahan 1983b
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)* F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZDM15*]	Yanisch-Perron *et al.* 1985

2.5.7 Preparation of competent cells

The protocol used in the preparation of competent cells is a modified version of Mandel and Higa (1970). LB medium (5ml) (pre-heated to 37°C) was inoculated with a single colony of *E. Coli* and incubated at 37°C (12-18h) while shaking. Fresh LB medium (9.9ml) was inoculated with 0.1ml of this culture and incubated for a further 2h under the same conditions. Cells were pelleted by centifugation (3000g, 5min at 4°C) and then resuspended in ice cold calcium chloride ($CaCl_2$) (5ml, 50mM). After incubation on ice (25min) the cells were pelleted as before and resuspended in ice cold $CaCl_2$ (1ml, 50mM). Competent cells were either kept on ice or mixed with sterile glycerol (40% v/v) and stored at -80°C.

2.5.8 Transformation of competent cells

Plasmid DNA (1-5μl) was added to 200μl of competent cells and incubated on ice (40min). After being heat shocked at 42°C for 90s cells were immediately placed on ice (2min). LB medium (800μl) (pre-warmed to 37°C) was added to the cells and incubated at 37°C (1h) while shaking. Transformed bacteria (200μl) were spread onto agar plates containing ampicillin (100μg/ml) (filter sterilised using

0.2μm filters). Remaining cells were pelleted by microcentrifugation (13,000 rpm, 3min), resuspended in LB medium (100μl) and spread onto separate plates. Colonies of transformed bacteria (ampicillin resistant) were grown overnight at 37°C.

2.5.9 Small-scale preparation of plasmid DNA (Miniprep)

LB medium (10ml) containing ampicillin (100μg/ml) was inoculated with a single colony of transformed *E.Coli* and incubated at 37°C (16h) while constantly shaking. Preparation of plasmid DNA from the bacterial culture was carried out using the Wizard SV™ Miniprep Kit according to the manufacturer's instructions. The concentration of DNA was calculated by measuring the absorption of light at wavelengths of 260nm and 280nm using a Hitachi U-1800 spectrophotometer.

2.5.10 Large-scale preparation of plasmid DNA (Maxiprep)

LB medium (12ml) containing ampicillin (100μg/ml) was inoculated with a single colony of transformed *E.Coli* and incubated at 37°C (7h) while constantly shaking. This was used to inoculate fresh LB medium/ampicillin (200ml) and grown overnight at 37°C while shaking. Plasmid DNA was prepared using the High Purity Plasmid Maxi Prep System according to the manufacturer's instructions (Marligen Biosciences). The concentration of DNA was calculated by measuring the absorption of light at wavelengths of 260nm and 280nm using a Hitachi U-1800 spectrophotometer.

2.5.11 Restriction endonuclease digestion of recombinant plasmid DNA

DNA (5μl) was mixed with the restriction endonuclease (8U) and its appropriate buffer in a total volume of 20μl. BSA (1x) was added where required. Buffers used in single and double digests were as recommended by the enzyme supplier (NEB). The reaction was incubated at 37°C (2h) for all enzymes used except SmaI for which the incubation temperature was 25°C. DNA fragments were analysed by agarose gel electrophoresis (Section 2.5.4).

2.5.12 Cloning of DNA oligonucleotides

2.5.12.1 Restriction endonuclease digestion of oligonucleotides

DNA oligonucleotides to be cloned into plasmid vectors were designed with the restriction sites for XhoI and Xma at the 5' and 3' ends respectively. Following annealing (Section 2.7.1.2.1) the oligonucleotides were digested with these restriction endonucleases (Section 2.5.11) to create 'sticky ends' and the products subjected to agarose gel electrophoresis (Section 2.5.4). The plasmid vector was also digested with the same endonucleases and subjected to agarose gel electrophoresis.

2.5.12.2 Gel extraction

DNA bands were excised from ethidium bromide stained agarose gels and purified using the QIAquick™ gel extraction kit according to the manufacturer's instructions (Qiagen). The efficiency of purification was assessed by electrophoresis on a 1% (w/v) agarose gel (Section 2.5.4).

2.5.12.3 Ligation

Vector (1μl) and insert DNA (1-7μl) was digested to create sticky ends (Section 2.5.12.1), gel purified (Section 2.4.12.2) and incubated at 16°C overnight, with T4 DNA ligase (1μl) and 1x T4 Ligase Buffer in a total volume of 10μl.

2.5.12.4 PCR screening of plated colonies

A single transformed bacterial colony was picked and added to a PCR mix containing GL1 and GL2 primers (composition as specified in Section 2.5.3 and Table 2.5), specific to regions either side of the multiple cloning site of pGL2 plasmids. The PCR reaction was carried out using a Peltier Thermal Cycler (PTC-200). Positive colonies were identified by resolving PCR products on a 1% (w/v) agarose gel (Section 2.5.4) and noting those of the correct size.

2.5.12.5 Automated DNA sequencing

Plasmid DNA was sequenced by Lark by automated fluorescent dye termination sequencing using the universal primers GL1 and GL2. For more information see http://www.lark.com.

2.6 PROTEIN ANALYSIS

2.6.1 Stock solutions

Table 2.7 Stock solutions for protein analysis

SOLUTION	COMPOSITION
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
Phosphatase-free whole cell extraction buffer	10mM Tris-HCl (pH 7.05), 50mM NaCl, 50mM NaF, 1% (v/v) Triton X-100, 30mM $Na_4P_2O_7$ (sodium pyrophosphate), 5μM $ZnCl_2$, 100μM Na_3VO_4 (sodium orthovanadate), 1mM DTT, 2.8μg/ml aprotinin, 2.5μg/ml each of leupeptin and pepstatin, 0.5mM benzamidine, 0.5mM PMSF
Nuclei extraction buffer A	10mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5mM PMSF, 1μg/ml pepstatin A, 10μg/ml aprotinin, 10μg/ml leupeptin, 10μg/ml type I-S soybean trypsin inhibitor
Nuclear extraction buffer C	25% (v/v) glycerol, 20mM HEPES (pH 7.9), 60mM KCl, 420mM NaCl, 1.5mM $MgCl_2$, 0.5mM PMSF in isopropanol, 10μg/ml type I-S soybean trypsin inhibitor 10 0.2mM EDTA, 0.5mM DTT, 10μg/ml aprotinin, 10μg/ml leupeptin
SDS-PAGE lower gel buffer	1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS
SDS-PAGE upper gel buffer	1M Tris-HCl (pH 6.8), 10% (w/v) SDS
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
SDS-PAGE running buffer	25mM Tris, 250mM glycine, 0.1% (w/v) SDS
Western blot transfer buffer	25mM Tris, 192mM glycine, 20% (v/v) methanol
10x Tris-buffered saline (TBS)	10mM Tris-HCl, 20mM NaCl, pH 7.4

2.6.2 Preparation of phosphatase-free whole cell protein extracts

Cells were washed once with PBS containing NaF (10mM) and Na_3VO_4 (sodium orthovanadate) (100μM) and subsequently resuspended in phosphatase-free whole cell extraction buffer (300-800μl) (described by Hipskind *et al.* (1994)). Cell suspensions were vortexed (45s) and debris was pelleted by microcentrifugation (10,000rpm, 10min) at 4°C. The supernatant was retained and stored at -80°C.

2.6.3 Preparation of nuclear protein extracts

Cells were pelleted by centrifugation (1000g, 5min) and washed three times by resuspension in ice-cold PBS and microcentrifugation (10,000rpm, 1min). After the final washing step, the supernatant was discarded and the pellet resuspended in ice-cold buffer A (50μl) (Table 2.7) and incubated on ice (5min). The cells were lysed by drawing 5 times through a Hamilton syringe and subjected to centrifugation (10,000rpm, 20s) at 4°C. The resulting nuclear pellet was gently resuspended in ice-cold buffer C (60μl) (Table 2.7) and incubated on ice (30min). After microcentrifugation at 4°C (10,000rpm, 5min) the supernatant was removed and stored at -80°C.

2.6.4 Determination of protein concentration

The concentration of total protein in whole cell extracts was determined using the Micro BCA Protein Assay Reagent Kit (Pierce) in accordance with the manufacturer's instructions. Briefly, protein samples were diluted in PBS and the concentration determined by a spectrophotometric reaction that occurs upon mixing with an equal volume of protein assay reagent (carried out in a 96-well micro-titre plate). The absorbance of each sample was read at 595nm using a Dynex Technologies MRX microplate reader and compared to a series of bovine serum albumin (BSA) standard dilutions in order to calculate the concentration.

2.6.5 Protein kinase B Activity Assay

PKB activity was determined using reagents provided in the Akt Kinase Assay Kit (non-radioactive) (Cell Signaling Technology). Whole cell extracts (250μg) were incubated with immobilised Akt antibody beads (20μl) overnight at 4°C. Beads were pelleted by microcentrifugation (13,000 rpm, 30s) and washed twice with 1x Cell Lysis Buffer (500μl) and twice with 1x Kinase Buffer (500μl). Beads were then resuspended in Kinase Buffer (50μl) supplemented with ATP (0.2mM) and GSK-3 Fusion Protein (1μg) The GSK-3 Fusion Protein contains residues corresponding to the peptide region surrounding the PKB phosphorylation site of

GSK-3α/β, Ser21/9, and acts as an artificial substrate. The kinase reaction was terminated by the addition of reducing solubilising solution (20μl). SDS-PAGE and western blot analysis was carried out on the supernatant fraction (Sections 2.6.7-2.6.9).

2.6.6 Immunoprecipitation

Whole cell extracts (150-300μg) were pre-cleared by the addition of Protein A/G Agarose beads (Santa-Cruz Biotechnology) (20μl) and incubation with gentle rolling at 4°C (1h), followed by microcentrifugation (13,000rpm, 3min) to remove the beads. The pre-cleared supernatant was mixed with an antibody raised against the desired protein (2μg/ml) and incubated overnight at 4°C with gentle rolling. The resulting protein-antibody complex was captured by the addition of Protein A/G Agarose (20μl) with gentle rotary mixing at 4°C (2h). The beads containing the captured immunocomplex were collected by microcentrifugation (13,000rpm, 3min) at 4°C and washed twice with phosphatase-free extraction buffer (without Triton X-100) (Table 2.7). They were then resuspended in 0.1M glycine (pH 2.5) (50-100μl) and incubated, with gentle rolling, at 4°C (10min) in order to displace the protein from the A/G Agarose. Beads were removed by microcentrifugation (9000rpm, 2min) and the immunoprecipitate used immediately for SDS-PAGE and western blot analysis (Sections 2.6.7-2.6.9).

2.6.7 Sodium dodecyl-sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

Table 2.8 Composition of SDS-PAGE gels for western blot analysis

GEL COMPOSITION	10% (W/V) SEPARATING GEL	15% (W/V) SEPARATING GEL	5% (W/V) STACKING GEL
Upper buffer	-	-	2.5ml
Lower buffer	5ml	5ml	-
Acrylamide:bisacrylamide (37.5:1)	5ml	7.5ml	1.25ml
ddH_2O	9.78ml	7.28ml	6.14ml
10% (w/v) APS	200μl	200μl	100μl
TEMED	20μl	20μl	10μl

SDS-PAGE was performed under reducing conditions following the method of Laemmli (1970). SDS-PAGE gels were prepared from stock solutions (Table 2.7) in the compositions shown in Table 2.8, using Mini-PROTEAN II™ slab electrophoresis cell apparatus (Bio-Rad Laboratories), assembled as described by the manufacturer. 1x Running Buffer was prepared containing 0.1% (w/v) SDS (Table 2.7). Whole cell protein extracts (10-100μg) or immunoprecipated lysates were separated by elecrophoresis on a 10% separating gel (for western blots with all antibodies with the exception of MCP-1 for which a 15% separating gel was used) after running through approximately 1cm of stacking gel (5%). Prior to loading, protein samples were mixed with reducing solubilising solution (in a ratio of 5:1) and boiled (3min).

2.6.8 Western Blotting

The separating gel and PVDF membrane (an appropriate size to fit the gel), previously activated in methanol, were equilibrated in transfer buffer (Table 2.7) at room temperature (15min). Electrophoretic transfer of proteins from the gel to the PVDF membrane was carried out using a TransBlot Electrophoretic transfer cell (BioRad Laboratories). The gel was placed in a blotting cassette adjacent to the PVDF membrane, between Whatman 3MM paper and the sponge pads of the transfer apparatus (pre-soaked in transfer buffer). This assembly was subjected to electroblotting in transfer buffer at 4°C, at a constant voltage of 15V for 12-18h or at 150V for 1h. Protein transferred to the PVDF membranes was immunodetected as described in Section 2.6.9.

2.6.9 Immunodetection of proteins

Blotted PVDF membranes were probed immunochemically in three stages: incubation with a blocking solution of 1x TBS containing 1-10% (w/v) skimmed milk and 0.05-0.2% (v/v) Tween-20; incubation with the primary antibody in 1x TBS, 1-5% (w/v) skimmed milk, 0.05-0.2% (v/v) Tween; and incubation with an appropriate HRP-conjugated secondary antibody in 1xTBS, 1-5% (w/v) skimmed milk, 0.05-0.1% (v/v) Tween. Membranes were washed three times after each incubation step with 1x TBS containing 0.05-0.1% (v/v) Tween. Conditions

relevant to each primary antibody are specified in Table 2.9. Detection of membrane bound antigen-antibody complexes was carried out using enhanced chemiluminescence (ECL) reagent as described in the manufacturer's instructions (Amersham Biosciences). Membranes were exposed to Kodak X-Ray film in a light proof cassette (Genetic Research Instrumentation) for varying exposure periods (3s-5min). X-ray films were developed using a Gevamatic 60 automatic developer (Agfa-Gevaert). The expected protein size was validated by comparison to standard Rainbow markers (Appendix II).

Table 2.9 Primary antibody binding conditions

PRIMARY ANTIBODY	DILUTION	% MILK (w/v)	% BSA (w/v)	INCUBATION (h)	SIZE (kDa)
STAT1 p84/91	1/1000	5	-	1	84/91
Phospho STAT1 Ser727	1/1000	5	-	Overnight[1]	84/91
Phospho STAT1 Tyr701	1/1000	5	-	Overnight[1]	84/91
p85	1/2000	3	-	Overnight[1]	85
CK2α	1/1000	5	-	1	42
CK2α'	1/1000	5	-	1	45
CK2β	1/1000	5	-	1	35
Sp1	1/1000	3	-	Overnight[1]	95/106
MCP-1	1/500	5	-	3	11
β-Actin	1/8000	1	-	1	42
PKB	1/1000	-	5	1	60
Phospho PKB Thr 308	1/1000	-	5	Overnight[1]	60
Phospho PKB Ser 473	1/1000	-	5	Overnight[1]	60
Phospho GSK3 (Akt Assay)	1/1000	-	5	Overnight[1]	30

[1]Overnight incubations carried out at 4°C

2.7 PROMOTER BINDING ANALYSIS

2.7.1 Electrophoretic Mobility Shift Assay (EMSA)

2.7.1.1 Stock solutions

Table 2.10 Stock solutions involved in EMSA analysis

SOLUTION	COMPOSITION
Dilution buffer	40mM KCl, 0.1mM EDTA
10× Binding buffer	340mM KCl, 50mM $MgCl_2$, 1mM DTT

2.7.1.2 Preparation of radiolabelled oligonucleotide probe DNA

2.7.1.2.1 Annealing of oligonucleotides

The sequences of the oligonucleotides used for EMSA analysis are shown in Table 2.11. 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.

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 2.11 Seqences of oligonucleotide probes for EMSA analysis

PROBE	ANNEALED OLIGONUCLEOTIDE
MCP-GC (-66/-33)	5' G GCA CCC TGC CTG ACT CCA CCC CCC TGG CTT A 3' 3' GGG ACG GAC TGA GGT GGG GGG ACC GAA T GTT G 5'
MCP-GAS (-166/-137)	5' GGCTTC CAC TTC CTG GAA ACA CCC GAG GG 3' 3' AAG GTG AAG GAC CTT TGT GGG CTC CC GAG 5'
Sp1	5' TAG ATT CGA TCG GGG CGG GGC GAG 3' 3' TAA GCT AGC CCC GCC CCG CTC CCG 5'
STAT1	5' CTT TTC CTG GAA TC 3' 3' GAA AAG GAC CTT AG 5'
NFκB	5' AGT TGA GGG GAC TTT CCC AGG C 3' 3' TCA ACT CCC CTG AAA GGG TCC G 5'
AP-1	5' GAT CCT TCG TGA CTC AGC GGG ATC CTT CGT GAC T 3' 3' A AGC ACT GAG TCG CCC TAG GAA GCA CTG AGT CGC C 5'

2.7.1.2.2 Radiolabelling of double-stranded oligonucleotides

Radiolabelling was carried out using the reagents supplied in the Megaprime™ Labelling Kit (Amersham Pharmacia Biotech) according the manufacturer's protocol. Double-stranded oligonucleotide (5ul, diluted to 35μl with ddH_2O) was incubated with 1x Labelling buffer, [α-^{32}P]-dCTP (3μl) and Klenow polymerase (2μl) at 37°C (30min). The reaction was stopped by the addition of 1× TE buffer (50μl) and separation of the radiolabelled probe from unincorporated nucleotides was carried out using a Sephadex G50 nick column. The column was equilibrated with 1× TE (9ml) and the reaction mix (100μl) loaded onto the column and eluted in 1× TE buffer (300μl) which was then discarded. The column was then further eluted with 1× TE (400μl) and the eluate collected and stored at -20°C.

2.7.1.3 DNA-protein binding reactions

Binding of radiolabelled double-stranded oligonucleotides to proteins present in phosphatase-free whole cell extracts (Section 2.6.2) was carried out according to Ramji *et al.* (1993). Briefly, phosphatase-free whole cell extracts (5μg) were diluted to a final volume of 13μl with dilution buffer (Table 2.7) and incubated with 1x binding buffer (Table 2.7) and 1μg/ml poly(dI-dC) (3μl) on ice (10min), to allow non-specific DNA binding to occur. Radiolabelled oligonucleotide (2μl) was then added and the reaction mixture was incubated at room temperature (20min).

2.7.1.4 Antibody supershift and competition binding studies

For supershift experiments antibody (2μg) was added to the DNA binding reaction. In competition binding studies, prior to addition of the radiolabelled probe, the binding reaction mixture (Section 2.7.1.3) was incubated with unlabelled probe (20min on ice) in molar excess of the radiolabelled probe by 250 fold.

2.7.1.5 Electrophoresis of DNA-protein complexes

Table 2.12 Composition of non-denaturing polyacrylamide gels for EMSA analysis

COMPONENT	4% (W/V) ACRYLAMIDE	6% (W/V) ACRYLAMIDE
Acrylamide: Bisacrylamide (29:1)	6ml	5ml
10x TBE	5ml	4ml
ddH_2O	42.5ml	30.5ml
10% APS	500µl	400µl
TEMED	50µl	40µl

Prior to electrophoresis 20% (w/v) Ficoll (5µl) was added to facilitate gel loading. DNA-protein complexes were resolved by electrophoresis on 4-6% (v/v) non-denaturing polyacrylamide gels (Table 2.12). Electrophoresis was carried out for 3-4h at 150V at 4°C using vertical gel apparatus (Scotlab) with 0.5× TBE as running buffer. Following electrophoresis, the gel was transferred to Whatmann 3MM paper and dried under vacuum using a Gel Dryer (Model 583, BioRad) at 80°C (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). X-ray films were developed using a Gevamatic 60 automatic developer (Agfa-Gevaert).

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2.7.2 Chromatin immunoprecipitation (ChIP) assay

2.7.2.1 Stock solutions

ChIP analysis was carried out using reagents supplied in the ChIP-IT^{TM} kit (Active Motif) according to the manufacturer's instructions with minor modifications as detailed. Solutions were made up as listed in Table 2.12.

Table 2.13 Solutions used for ChIP Assay

SOLUTION	COMPOSITION
PBS/PMSF	1x PBS; 0.5mM PMSF
Glycine Stop-Fix Solution	1x Glycine Buffer, 1xPBS
Lysis Buffer	1x Lysis Buffer; 5µl Phosphatase Inhibitor Cocktail (PIC)/ml); 0.5mM PMSF
Shearing Buffer	1x Shearing Buffer; 5µl PIC/ml)
ChIP IP Buffer	1x IP Buffer; 5µl PIC/ml)
Wash Buffer 1	1x Wash Buffer 1; 1µl PIC/ml)
Wash Buffer 2	1x Wash Buffer 1; 1µl PIC/ml)
ChIP Elution Buffer	1% SDS; 50µM $NaHCO_3$

2.7.2.2 Formaldehyde crosslinking

Cells (approximately 3 x 10^5 in a 75cm^2 flask) were fixed by the addition of 37% (v/v) formaldehyde to the culture medium (27µl 37% (v/v) formaldehyde/ml medium) followed by incubation at room temperature (10min) while rocking gently. Cells were washed in PBS/PMSF (5ml) and incubated in Glycine Stop-Fix Solution (30ml) (5min) while rocking gently. Following this cells were washed once in PBS/PMSF (5ml), resuspendend in ice-cold Lysis Buffer (0.75ml) and incubated on ice (30min). The cell suspension was drawn ten times through a Hamilton syringe and the nuclei pelleted by microcentrifugation (5000rpm, 10min) at 4°C. Nuclei were resuspended in Shearing Buffer (0.5ml) and subjected to sonication as described below.

2.7.2.3 Chromatin shearing

Chromatin shearing was achieved through sonication using a Sonics Vibracell™ Ultrasonic Processor with a 3mm microtip. Sonication was carried out on ice using optimised shearing conditions (determined in Section 4.4.2.2) consisting of twelve pulses of 30s at 24% power, separated by 30s pauses. Following microcentrifugation (13,000rpm, 12min) at 4°C the supernatant fraction, containing sheared chromatin, was retained. Shearing efficiency was analysed by electrophoresis of sheared DNA through a 2% (w/v) agarose gel subsequent to the reversal of cross-links (Section 2.7.2.5).

2.7.2.4 Chromatin immunoprecipitation

Sheared chromatin (50μl/reaction) was diluted in ChIP IP Buffer (60μl) and precleared by incubation with Protein G beads (100μl) (1h) with rotation at 4°C. Following microcentrifugation (4000rpm, 2min) the precleared supernatant was incubated with the relevant antibody (4μg) overnight at 4°C. An aliquot (10μl) of precleared chromatin was retained for use as an "Input" sample. Protein G beads (100μl) were added to the antibody/chromatin and incubated at 4°C (1.5h). The beads were then washed once with ChIP IP Buffer; four times with Wash Buffer 1; once with Wash Buffer 2; and twice with Wash Buffer 3 (supplied ready-to-use). For each wash, beads were pelleted by centrifugation (4000rpm, 2min) and incubated with Wash Buffer for 2min with rotation. DNA was collected from the Protein G beads by incubation in ChIP Elution buffer (50μl) (15min) with rotation. This step was performed twice and aliquots pooled to give a final elution volume of 100μl.

2.7.2.5 Reversal of crosslinking and DNA purification

ChIP elutions (100μl) and Input DNA (diluted to 100μl in ddH_2O) were incubated overnight at 65°C in the presence of NaCl (0.2M) and RNaseA (0.1μg/μl). Subsequently, the samples were treated with Proteinase K (0.1μg/μl) at 42°C (1.5h) in the presence of EDTA (10mM) and Tris-Cl (pH6.5) (20mM). DNA was

purified according to the Active Motif protocol using the mini-columns provided and eluted in a final volume of 100µl ddH_2O.

2.7.2.6 PCR analysis

PCR analysis was carried out using conditions and primers as specified in Table 2.5 and products were separated by agarose gel electrophoresis on a 2% (w/v) agarose gel (Section 2.5.4).

2.8 MICROARRAY

2.8.1 SuperArray Oligo GEArray DNA Microarrays

Microarray experiments were performed using Oligo GEArray™ Mouse Atherosclerosis Microarrays supplied by SuperArray Bioscience Corporation. The arrays are based on a Nylon matrix platform containing gene-specific oligonucleotides (60bp) (Appendix V). In brief, a RNA sample is converted to labelled cRNA that is hydrised to the array membrane and detected by a chemiluminescent method. Procedures were carried out using the reagents supplied in the Oligo GEArray™ Reagent Kit according to the relevant protocols.

2.8.2 Stock solutions

Table 2.14 Stock solutions used for microarray based experiments

SOLUTION	COMPOSITION
20x SSC	3M NaCl; 0.3M sodium citrate; HCl pH 7.0
Wash Solution 1	2x SSC; 1% (w/v) SDS
Wash Solution 2	0.1x SSC; 0.5% (w/v) SDS

2.8.3 Synthesis of labelled cRNA

Total RNA (3μg) (Section 2.5.2) was converted to a labelled target cRNA probe using the TrueLabeling-AMP™ Linear RNA Amplification Kit (SuperArray Bioscience Corporation) according to the manufacturer's protocol. Briefly this involved a cDNA synthesis reaction followed by an overnight cRNA synthesis and amplification reaction incorporating biotinylated-dUTP. Labelled cRNA was purified using the ArrayGrade cRNA Cleanup™ Kit (SuperArray Bioscience Corporation) according to the instructions provided by the manufacturer. The quality and concentration of cRNA obtained was calculated by measuring the absorption of light at wavelengths of 260nm and 280nm with a Hitachi U-1800 spectrophotometer. Labelled cRNA was also analysed by resolving 1μg on a 1% (w/v) agarose gel (Section 2.5.4) and by dot blot (Section 2.8.4).

2.8.4 Dot blot to analyse cRNA labelling efficiency

Serial dilutions of labelled cRNA were prepared in the ratios: 1/20; 1/80; 1/320; 1/1280; 1/5120. Dilutions (1μl) were dotted onto nitrocellulose membrane (preactivated in methanol) and allowed to dry. Chemiluminescent detection was carried as described in Section 2.8.6. The detection of a signal at a dilution of 1/1280 was considered to be sufficient for microarray analysis.

2.8.5 Hybridisation

Hybridisation procedures were carried out in a Hybaid™ rotary oven at a rotation speed of 20rpm. Array membranes were prehybridised in GEAhyb™ Hybridisation solution (2ml) with continuous rotation at 60°C (2h). Following this, biotin-labelled cRNA target (6μg) was added to GEAhyb™ Hybridisation solution (0.75ml) and incubated with the array membrane overnight with continuous rotation at 60°C. Membranes were washed once with Wash Solution 1 (Table 2.14) (15min) and once with Wash Solution 2 (Table 2.14) (15 min), both at 60°C with continuous agitation.

2.8.6 Chemiluminescent detection

Chemiluminscent detection was performed using the Chemiluminescent Detection Kit according to the manufacturer's protocol (SuperArray Bioscience Corporation). Membranes were exposed to Kodak X-Ray film in a light proof cassette (Genetic Research

Instrumentation) for varying exposure periods (3s-15min). X-ray films were developed using a Gevamatic 60 automatic developer (Agfa-Gevaert).

2.8.7 Data analysis

The data analysis protocol is summarised in Figure 2.1. Microarray images were scanned from X-ray film using a HP Scanjet 5470c and saved as files in TIFF format. The average spot density for each gene, taken as the expression value (E) for that gene (E; gene), was measured using GEArray™ Expression Analysis Software (SuperArray Bioscience Corporation) accessible at http://GEASuite.superarray.com. As few genes were detectable at low exposures, yet the signal for others became saturated at high exposures, it was necessary to use data over a range of exposure times and normalise data between the signals (Section 2.8.7.2). Between 7-10 images obtained from a range of membrane exposure times were analysed to create a dataset that was exported to Microsoft Excel for analysis.

2.8.7.1 Background subtraction

A background reading (B) for each array was determined by taking the mean value of E for the dataset, excluding control genes, which was subtracted from E; gene. An exposure was selected for each gene whereby E was above the background level but below the saturation point (S) for that array. S was defined as the value of E at which increasing exposure times did not increase the value measured.

2.8.7.2 Data normalisation

In order to normalise data between different arrays in the same experiment, the relative value of E; GAPDH was compared between arrays, at an exposure time for which S was not reached in any array. As E; GAPDH reached saturation at exposures in which only a small number of other genes showed measurable expression, a second gene (C) was selected in the array representing the "Untreated" (UT) sample, for which E spanned all exposures without reaching S. E for all other genes was normalised to this gene at the relevant exposures. Where other arrays ("Treated", T) were compared to array UT, C values were multiplied by the ratio: T; (E-B) GAPDH: UT; (E-B) GAPDH, to give C2 to which genes in array T were normalised. This gives values of E(norm) for each gene that can then be compared.

2.8.7.3 Statistical analysis

The fold induction (F), expressed by: [T: E(norm); gene]/[UT: E(norm); gene], was calculated for each gene. The $\log_2$ F was calculated so that genes for which there was no change in expression were given a value of zero; those for which expression was reduced by half were given a value of -1; and for a two-fold induction, +1. The mean $\log_2$ F was taken from three independent experiments and the standard deviation (SD) of $\log_2$ F calculated. A t-test was carried out on this data (Appendix IV) to determine whether there was a significant change in expression between T and UT. A P-value of < 0.05 was accepted as significant. A change in expression for which P < 0.1 was accepted as significant subject to PCR validation (denoted by *). Unchanged expression was reported where 0.5 < F < 1.5 with SD > 1.

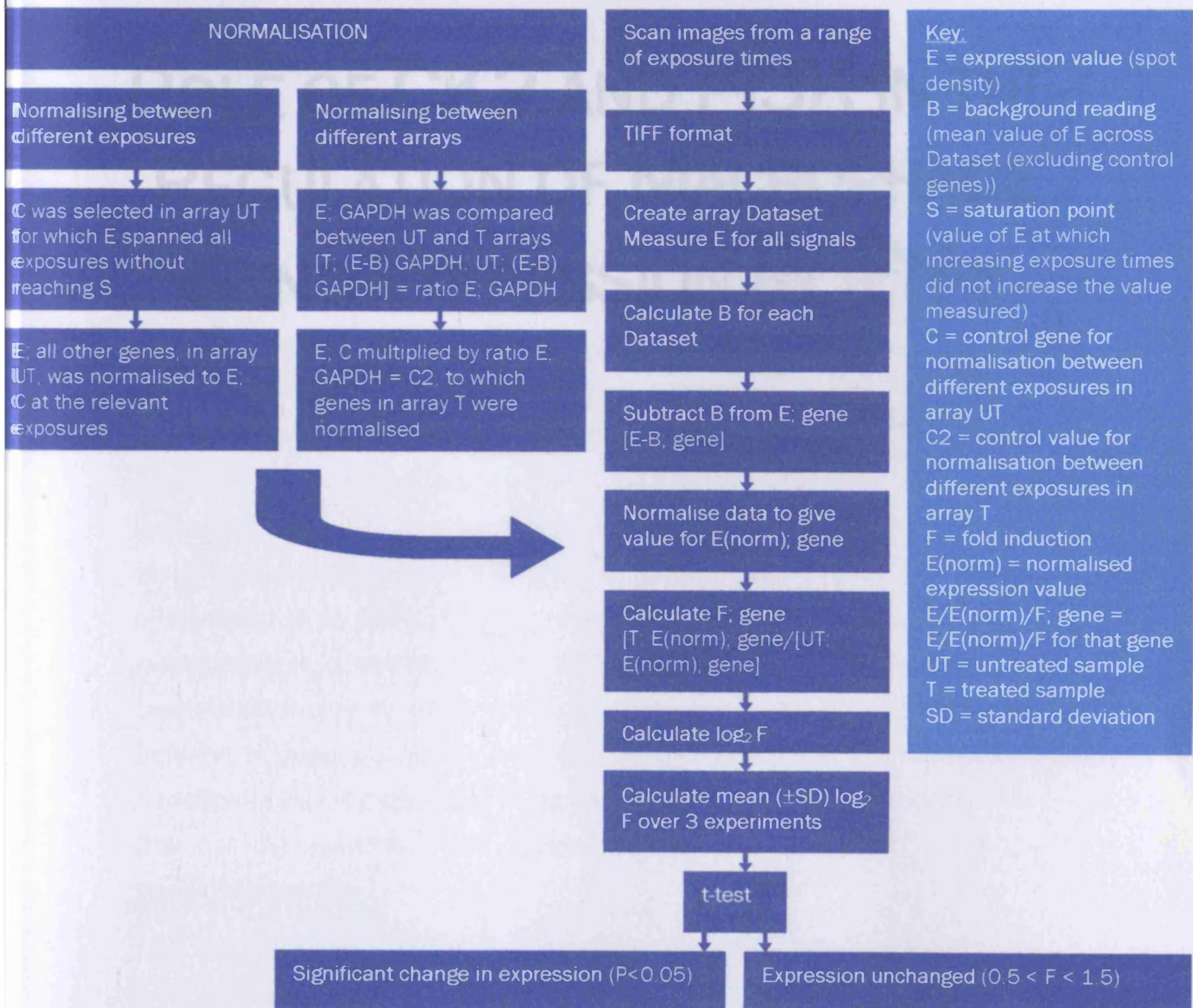


Figure 2.1 Microarray data normalisation

CHAPTER THREE:

ROLE OF CK2 AND PI3K IN THE REGULATION OF MACROPHAGE GENE EXPRESSION BY IFN-γ

CHAPTER 3. ROLE OF CK2 AND PI3K IN THE REGULATION OF MACROPHAGE GENE EXPRESSION BY IFN-γ

3.1 INTRODUCTION

The cytokine IFN-γ modulates the expression of numerous genes, many of which are involved in the progression of the atherosclerotic lesion. Studying the signalling pathways through which IFN-γ regulates the expression of these genes could be of benefit in the search for therapeutic targets for the disease.

3.1.1 Experimental strategy

It was decided that the work presented here would be carried out predominantly in macrophages as these cells, known to be responsive to IFN-γ, are a key cellular component of the atherosclerotic plaque (Section 1.4.4). The transformed murine macrophage cell line, J774.2 (Section 2.3.1), was selected as a model system. Of the commercially available monocyte/macrophage cell lines, cells of the J774.2 line most closely represent differentiated macrophages (Ralph and Nakoinz 1977, Ralph *et al.* 1975, Ralph and Nakoinz 1975). In comparison to primary monocyte-derived macrophages, the cell line provides a more homogeneous system and allows for the extraction of a greater yield of RNA or protein. The J774.2 cell line has been used successfully to model macrophage responses to IFN-γ in our laboratory, giving data that has subsequently been replicated in primary cells and other macropahge systems (Mead *et al.* 2003, Hughes *et al.* 2002, Tengku-Muhammad *et al.* 1998, Tengku-Muhammad *et al.* 1996). Studying signalling mechanisms in a murine cell line not only allows comparison to the common mammalian model for atherosclerosis development, the apoE-null mouse, but patterns in gene expression are also well-conserved with human responses. Additionally, key findings were to be studied in human primary macrophages to rule out the possibility that observations were a peculiar property of the transformed cell line.

Having previously established, in our laboratory, a role for the protein kinases CK2 and PI3K in the IFN-γ-mediated regulation of LPL and ICER gene expression (Mead *et al.* 2003, Hughes *et al.* 2002, Tengku-Muhammad *et al.* 1999b, Evans, S., personal communication), the primary aim of the research presented in this chapter was to further analyse the function of these kinases in IFN-γ signalling. This was to be achieved initially by studying the effect of specific pharmacological inhibitors of these mediators on the induction of a selection of genes by IFN-γ. By this we hoped to gain information about the role of PI3K and CK2 in alternative pathways controlling the regulation of gene expression by the cytokine. Data from these experiments would then be confirmed with the use of dominant negative mutants and the mechanisms involved in promoter activation studied in more detail (see Figure 3.1 for overall strategy).

3.1.2 Selection of genes for study

In order to achieve the aims detailed above it was first necessary to select genes of interest for further study. We looked for a range of genes, that have previously been found to be inducible by IFN-γ in at least one cellular system, that were potentially regulated by the cytokine through distinct signalling mechanisms.

The JAK-STAT pathway is the most widely accepted signalling mechanism activated by IFN-γ (Section 1.7). However, studies by Ramana *et al.* (2001, 2002) have highlighted the existence of potential STAT1-independent pathways in the regulation of gene expression by IFN-γ. They have shown that STAT1-deficient cells are able to support the regulation of a number of genes in response to IFN-γ treatment (Ramana *et al.* 2002, Gil *et al.* 2001, Ramana *et al.* 2001) (Section 1.8.1). Among these is the proatherogenic chemokine MCP-1, discussed in more detail in Section 1.6.

The induction of other genes by IFN-γ, including suppressor of cytokine signalling (SOCS)-1, was found to be possible in wild type cells only (Ramana *et al.* 2002). SOCS-1 acts as a negative regulator of several cytokine pathways, in particular the JAK-STAT pathway. Knock-out of the SOCS-1 gene in mice results in neonatal lethality due to a complex inflammatory disease resulting in fatty degeneration of

the liver. This pathology is thought to be caused by tissue hypersensitivity to IFN-γ as SOCS-1/IFN-γ double knock-out mice do not suffer from neonatal disease (Cornish *et al.* 2003, Alexander 2002, Alexander *et al.* 1999). SOCS-1 has also been shown to localise to the atherosclerotic lesion in apoE-null mice (Tang *et al.* 2005) and is likely to modulate the inflammatory component of the disease.

As discussed previously, a recent study in our laboratory has demonstrated that the expression of the transcriptional repressor ICER is upregulated in response to IFN-γ through the activation of the CREB family of transcription factors by CK2 (Mead *et al.* 2003) (Sections 1.10 and 1.11). While transcriptional activation by IFN-γ has been extensively studied, far less is known about the repression of gene expression by this cytokine. The upregulation of ICER may represent a novel mechanism for transcriptional repression in macrophages by IFN-γ. Several genes that are implicated in atherosclerosis development are downregulated in response to IFN-γ (*e.g.* SR-A, VLDL-R, and LRP (Schulz *et al.* 2003, Kosaka *et al.* 2001, LaMarre *et al.* 1993, Geng and Hansson 1992) and the signalling pathways involved are of potential therapeutic interest. The induction of ICER mRNA expression by IFN-γ was not found to be attenuated in the presence of the pharmacological inhibitor of JAK2, AG490, indicating that a novel JAK-independent pathway may be involved that leads to the regulation of CREB/CREM/ATF binding activity.

Due to the potentially differential mechanisms involved in the regulation of MCP-1, SOCS-1 and ICER expression by IFN-γ, these three genes were selected for further study.

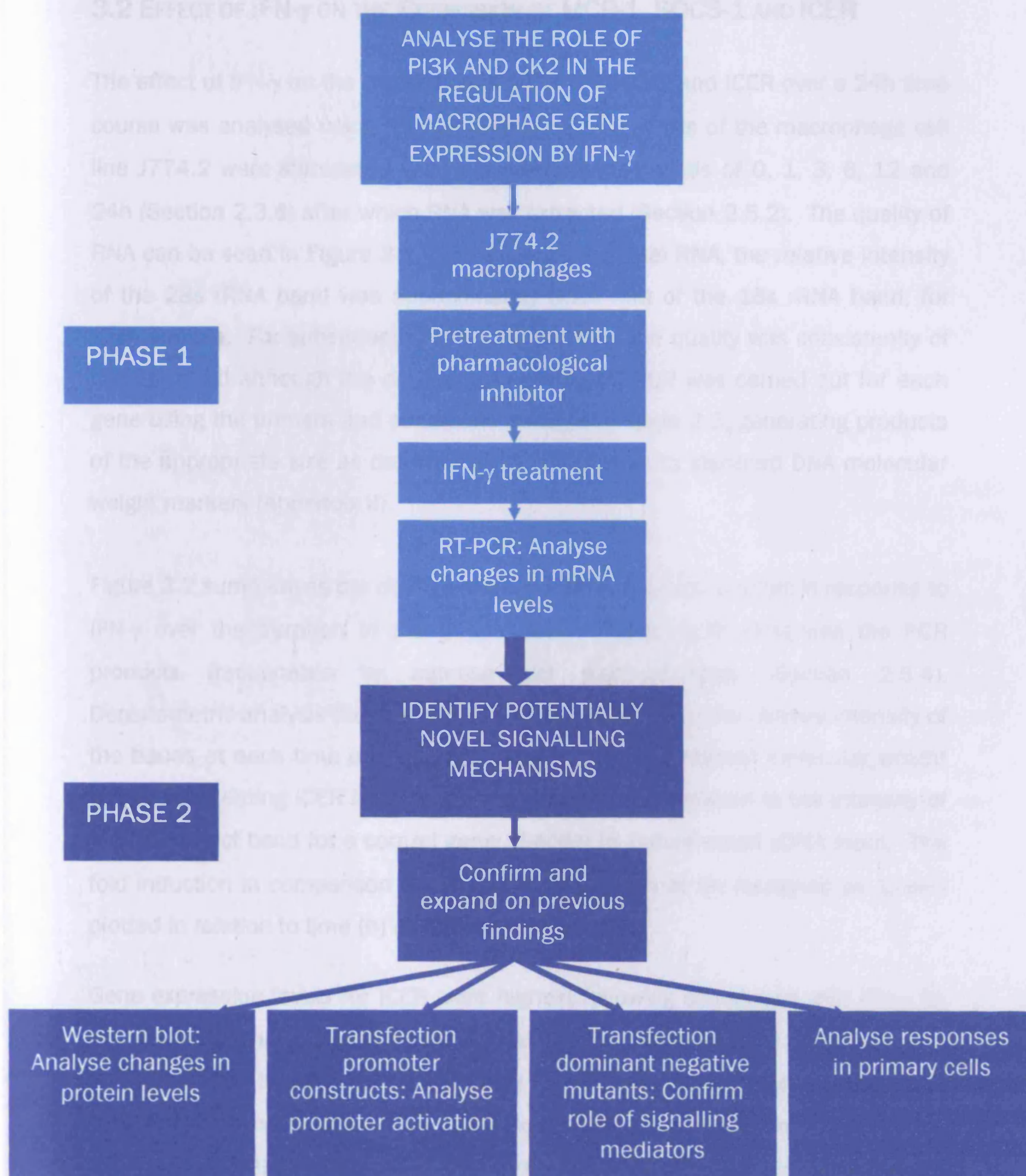


Figure 3.1 Experimental strategy

The aim of Phase 1 of the experimental strategy was to analyse the role of the kinases PI3K and CK2 in the regulation of the expression of a selection of genes by IFN-γ using specific pharmacological inhibitors. The data from this set of experiments were to be used to identify potentially novel signalling mechanisms in the regulation of one or several of the selected genes. Phase 2 of the strategy involved the confirmation and expansion of previous findings.

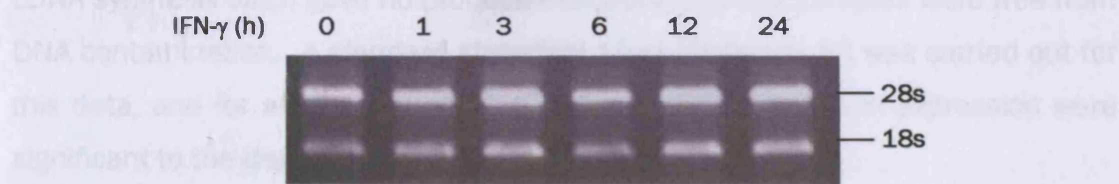
3.2 EFFECT OF IFN-γ ON THE EXPRESSION OF MCP-1, SOCS-1 AND ICER

The effect of IFN-γ on the expression of MCP-1, SOCS-1 and ICER over a 24h time course was analysed using RT-PCR (Section 2.5.3). Cells of the macrophage cell line J774.2 were stimulated with murine IFN-γ for periods of 0, 1, 3, 6, 12 and 24h (Section 2.3.6) after which RNA was extracted (Section 2.5.2). The quality of RNA can be seen in Figure 3.2A. As expected for total RNA, the relative intensity of the 28s rRNA band was approximately twice that of the 18s rRNA band, for each sample. For subsequent work involving RNA the quality was consistently of this standard although the data is not shown. RT-PCR was carried out for each gene using the primers and conditions detailed in Table 2.5, generating products of the appropriate size as determined by comparison to standard DNA molecular weight markers (Appendix II).

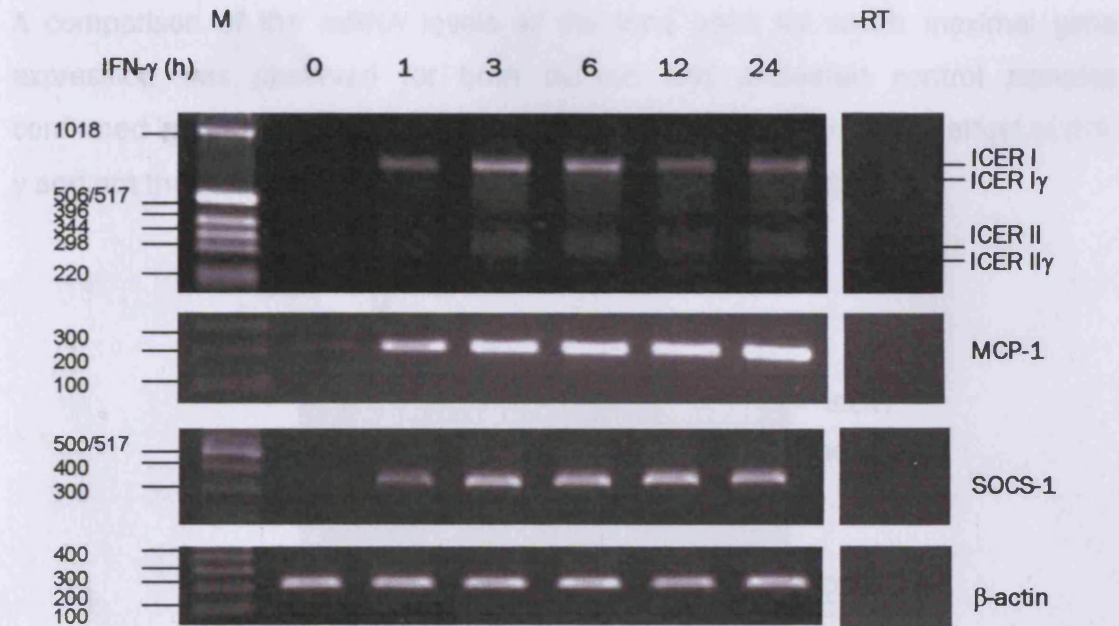
Figure 3.2 summarises the change in mRNA levels for each species in response to IFN-γ over the duration of the time course. Figure 3.2B illustrates the PCR products fractionated by agarose gel electrophoresis (Section 2.5.4). Densitometric analysis (Section 2.5.5) was carried out and the relative intensity of the bands at each time point (in the case of ICER, the highest molecular weight band, representing ICER I, was used) was calculated in relation to the intensity of a PCR product band for a control gene (β-actin) to ensure equal cDNA input. The fold induction in comparison to the mRNA expression at 0h (assigned as 1) was plotted in relation to time (h) (Figure 3.2C).

Gene expression levels for ICER were highest following stimulation with IFN-γ for 3h, after which point there was a marginal decrease (Figures 3.2B and 3.2C). It is also clear from these results that ICER I is the major mRNA expressed in these cells following exposure to IFN-γ. Similar to ICER, the expression of MCP-1 was highest after a 3h treatment period. In this case however, the level of expression did not decrease for subsequent time points (Figures 3.2B and 3.2C). SOCS-1 shows a similar expression profile in response to IFN-γ with mRNA levels reaching a maximum after 3h treatment and remaining at this level for the duration of the time course (Figures 3.2B and 3.2C).

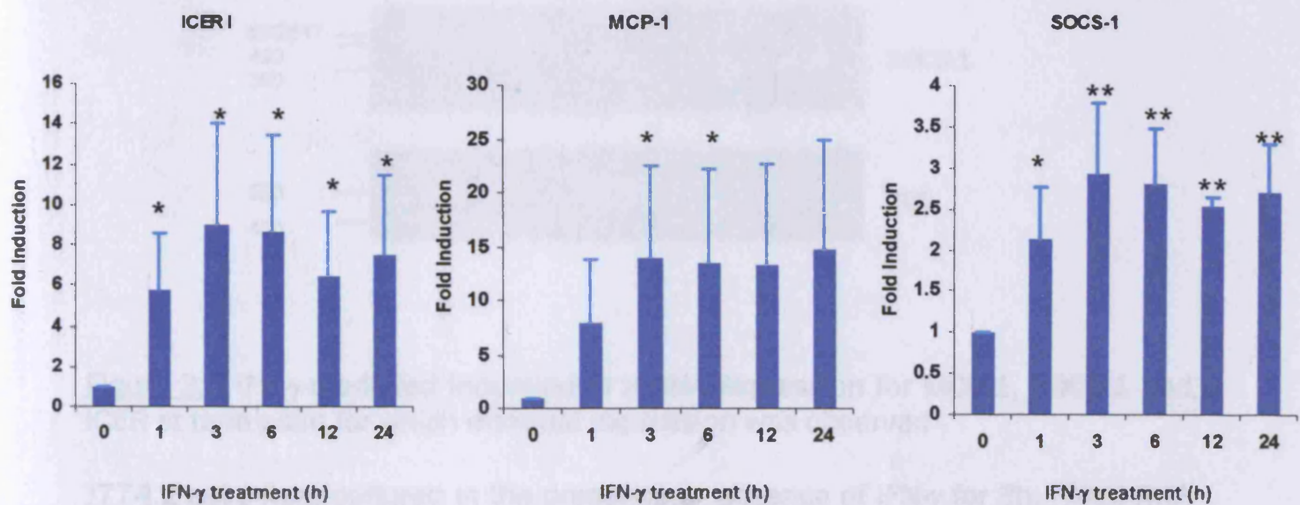
A.



B.



C.



A negative (–)RT reaction (for which reverse transcriptase was not included in the cDNA synthesis step) gave no product, indicating that the samples were free from DNA contamination. A standard statistical t-test (Appendix IV) was carried out for this data, and for all subsequent densitometry, and changes in expression were significant to the degree indicated.

A comparison of the mRNA levels at the time point for which maximal gene expression was observed for both treated and untreated control samples confirmed in all cases that the increase in expression was due to the effect of IFN-γ and not the status of the cells at that time point (Figure 3.3).

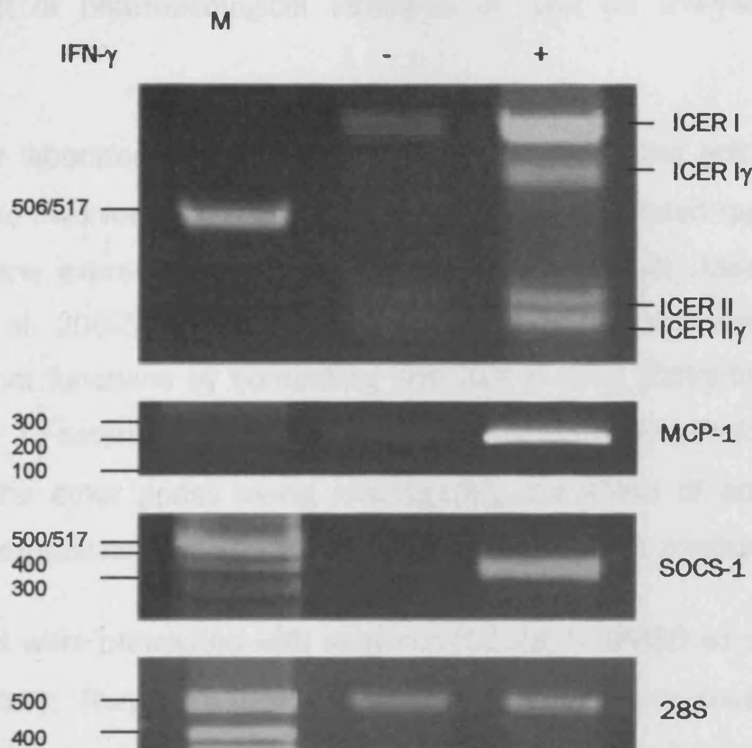


Figure 3.3 IFN-γ-mediated induction in mRNA expression for MCP-1, SOCS-1 and ICER at time point for which maximal expression was observed

J774.2 cells were cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers ICER I/II; MCP-1; SOCS-1; 28S. The amplification products were analysed by agarose gel electrophoresis. The position of the appropriate product and the size of the DNA markers are indicated.

3.3. EFFECT OF PHARMACOLOGICAL INHIBITORS ON IFN-γ-STIMULATED GENE EXPRESSION

The profile of induction for MCP-1, SOCS-1 and ICER by IFN-γ over time established an optimal treatment time of 3h for which maximal levels of mRNA were observed for each of these genes. Further studies aiming to determine components of the signalling pathways involved in this response were carried out with cells stimulated with IFN-γ for this time. Specific pharmacological inhibitors were employed to study the role of various signalling mediators at concentrations widely used in published literature (Table 2.2).

3.3.1 Effect of pharmacological inhibition of CK2 on IFN-γ-stimulated gene expression

Work in our laboratory has shown that IFN-γ stimulates the activity of CK2 and indicated the involvement of this kinase in the IFN-γ-mediated regulation of ICER and LPL gene expression in macrophages (Section 1.10) (Mead *et al.* 2003, Hughes *et al.* 2002). These experiments utilised the selective CK2 inhibitor apigenin, that functions by competing with ATP binding (Critchfield *et al.* 1997) (Table 2.2). To determine whether CK2 has a role in the IFN-γ-inducible regulation of any of the other genes being investigated, the effect of apigenin on IFN-γ stimulated expression was studied using ICER as a positive control.

J774.2 cells were pretreated with apigenin (10μM) or DMSO as a vehicle control (Section 2.3.7). They were then cultured in the presence or absence of IFN-γ for 3h, the time at which MCP-1, SOCS-1 and ICER exhibit maximal induction. Following treatment, RNA was extracted and RT-PCR analysis carried out for each gene as before. Figure 3.4 shows the PCR products for each gene resolved by agarose gel electrophoresis (Figure 3.4A) and the band densities normalised to the expression of the control gene β-actin (Figure 3.4B). For each of the selected genes IFN-γ can be seen to have stimulated an increase in mRNA levels as expected. When combined with IFN-γ treatment, apigenin reduced gene expression levels, suggesting that CK2 is involved in the IFN-γ-induced signalling pathways for all of these genes.

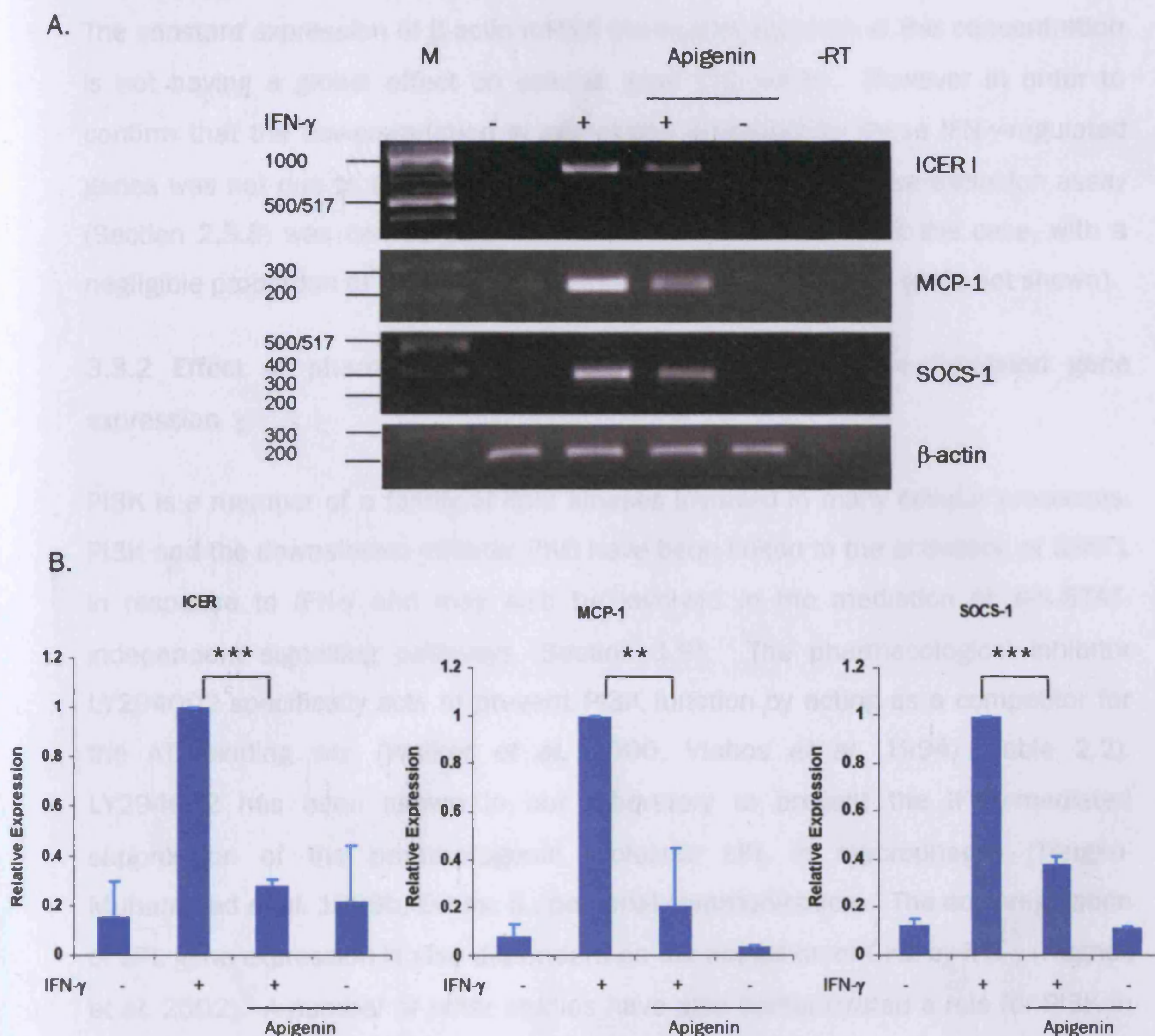


Figure 3.4 Effect of apigenin on the induction of ICER, MCP-1 and SOCS-1 by IFN-γ

J774.2 cells were pretreated with apigenin (10μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers: ICER I/II; MCP-1; SOCS-1; β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The position of the appropriate product and the size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) of the appropriate gene normalised to the expression of β-actin as determined by densitometric analysis from three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). **P<0.01; ***P<0.001

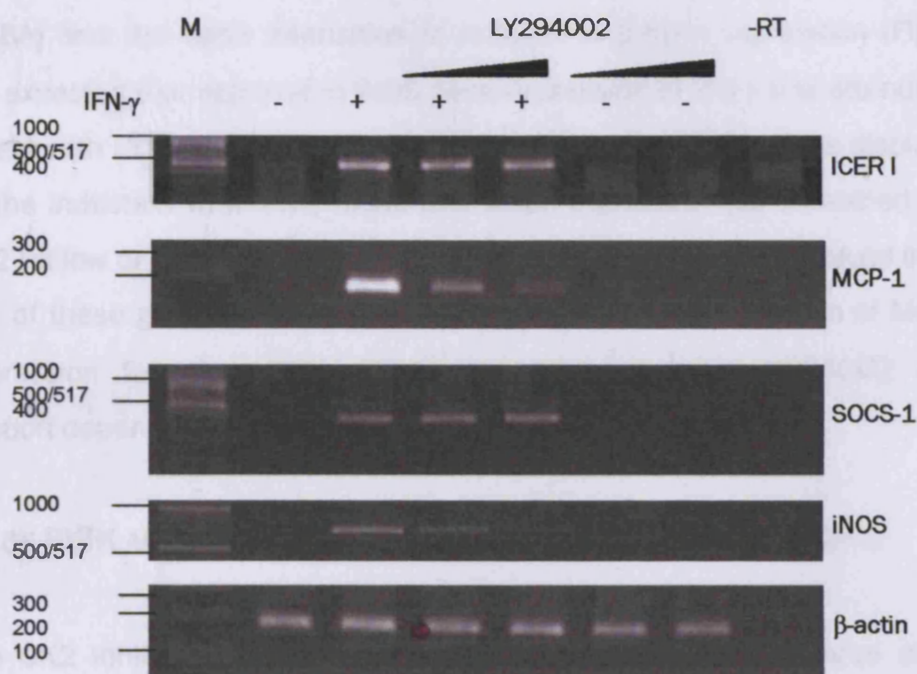
The constant expression of β-actin mRNA shows that apigenin at this concentration is not having a global effect on cellular gene expression. However in order to confirm that the downregulation in expression observed for these IFN-γ-regulated genes was not due to a toxic effect of the inhibitor, a trypan blue exclusion assay (Section 2.3.8) was carried out. It was found that this was not the case, with a negligible proportion of cells dying under all treatment conditions (data not shown).

3.3.2 Effect of pharmacological inhibition of PI3K on IFN-γ-stimulated gene expression

PI3K is a member of a family of lipid kinases involved in many cellular processes. PI3K and the downstream effector PKB have been linked to the activation of STAT1 in response to IFN-γ and may also be involved in the mediation of JAK-STAT-independent signalling pathways (Section 1.9). The pharmacological inhibitor LY294002 specifically acts to prevent PI3K function by acting as a competitor for the ATP-binding site (Walker *et al.* 2000, Vlahos *et al.* 1994) (Table 2.2). LY294002 has been shown in our laboratory to prevent the IFN-γ-mediated suppression of the proatherogenic molecule LPL in macrophages (Tengku-Muhammad *et al.* 1999b, Evans, S., personal communication). The downregulation of LPL gene expression is also dependent on the activation of CK2 by IFN-γ (Hughes *et al.* 2002). A number of other studies have also demonstrated a role for PI3K in the IFN-γ-mediated regulation of certain responses as discussed in Section 1.9. The effect of PI3K inhibition by LY294002 on the IFN-γ-mediated induction of MCP-1, SOCS-1 and ICER was therefore investigated. Hwang *et al.* (2004) have previously demonstrated that the IFN-γ-mediated induction of iNOS expression was attenuated in the presence of LY294002. The efficacy of the inhibitor was determined by reproducing the effect on the induction of iNOS expression by IFN-γ.

J774.2 cells were pre-treated with LY294002 (5μM and 20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h, after which RNA was extracted. RT-PCR was carried out for each gene as before and for iNOS using the primers specified in Table 2.5.

A.



B.

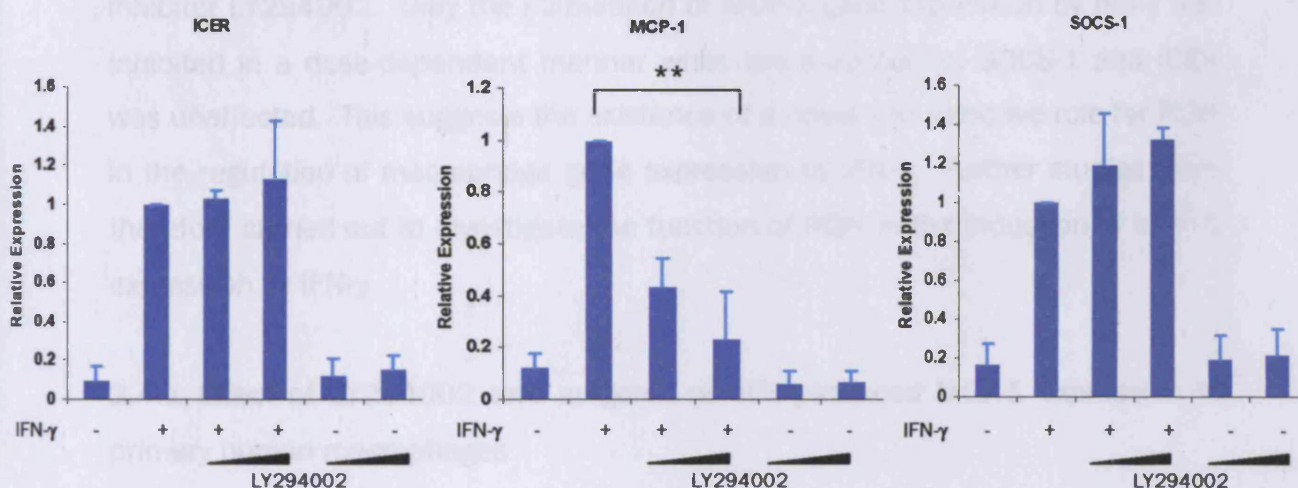


Figure 3.5 Effect of LY294002 on the induction of ICER, MCP-1 and SOCS-1 expression by IFN-γ

J774.2 cells were pretreated with LY294002 (5μM and 20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers: ICER I/II; MCP-1; SOCS-1; iNOS, β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The position of the appropriate product and the size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) of the appropriate gene normalised to the expression of β-actin as determined by densitometric analysis from three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). **P<0.01

Figure 3.5 shows the PCR products using primers specific to each mRNA species (Figure 3.5A) and the band intensities in relation to β-actin expression (Figure 3.5B). As expected the response in iNOS gene expression to IFN-γ was attenuated by treatment with LY294002. For ICER I and SOCS-1 no difference was displayed between the induction with IFN-γ alone and when treatment was combined with LY294002 (at low or high concentration) indicating that PI3K is not involved in the regulation of these genes in response to IFN-γ. In contrast stimulation of MCP-1 gene expression following IFN-γ treatment is inhibited by LY294002 in a concentration dependent manner as illustrated in Figure 3.5.

3.4 ROLE OF PI3K AND PKB IN THE IFN-γ-MEDIATED INDUCTION OF MCP-1

While the CK2 inhibitor apigenin attenuated the induction of all three of the selected genes by IFN-γ, the genes were differentially responsive to the PI3K inhibitor LY294002. Only the stimulation of MCP-1 gene expression by IFN-γ was inhibited in a dose-dependent manner while the induction of SOCS-1 and ICER was unaffected. This suggests the existence of a novel and selective role for PI3K in the regulation of macrophage gene expression by IFN-γ. Further studies were therefore carried out to investigate the function of PI3K in the induction of MCP-1 expression by IFN-γ.

3.4.1 Effect of LY294002 and apigenin on IFN-γ-induced MCP-1 expression in primary human macrophages

To rule out the possibility that the results obtained are a peculiar property of the transformed J774.2 cell line, the effects of IFN-γ and the inhibitors LY294002 and apigenin on MCP-1 gene expression in human primary monocyte-derived macrophages were investigated. Primary monocytes were isolated from human blood and allowed to differentiate in culture over seven days (Section 2.3.4). Macrophages were pretreated with apigenin (20μM), LY294002 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h, after which RNA was extracted. RT-PCR was carried out using primers specific for human MCP-1 and β-actin (Table 2.5) and products resolved by agarose gel electrophoresis (Figure 3.6A). The density of the product bands as

determined by densitometric analysis was normalised to those for β-actin as illustrated in Figure 3.6B. As shown in J774.2 cells (Figure 3.4-3.5), apigenin and LY294002 both inhibited the induction of MCP-1 expression by IFN-γ in human monocyte-derived macrophages.

A.

B.

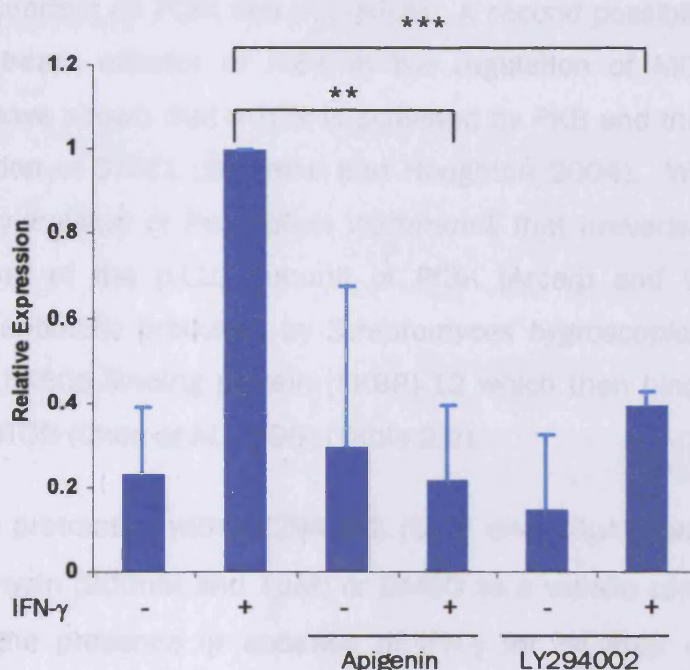


Figure 3.6 Inhibition of IFN-γ-mediated induction of MCP-1 mRNA expression by LY294002 and apigenin in primary monocyte-derived macrophages

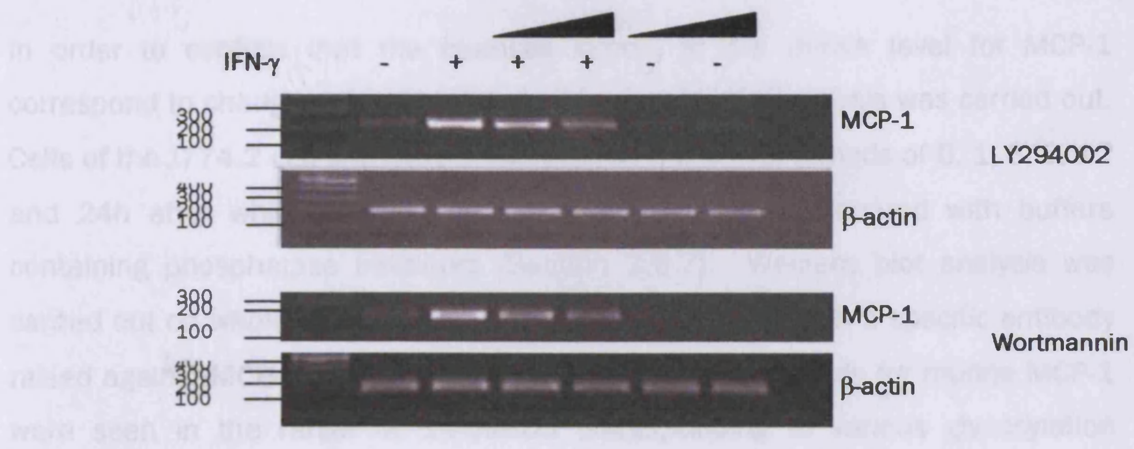
Primary monocyte-derived macrophages were pre-treated with apigenin (20μM), LY294002 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against hMCP-1 and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The position of hMCP-1 and the size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). Results are representative of two independent experiments. **P<0.01; ***P<0.001.

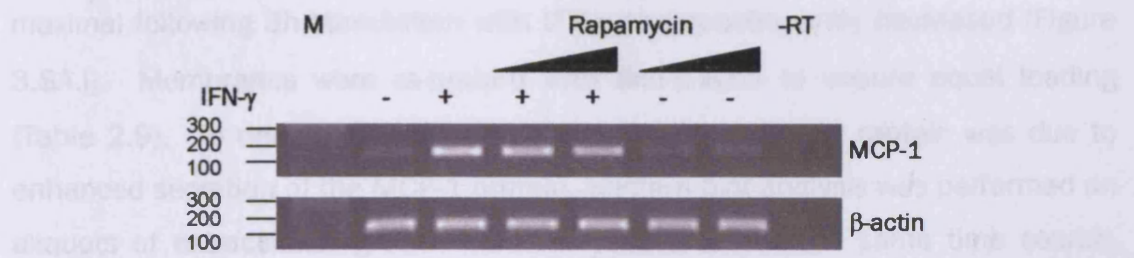
3.4.2 Effect of wortmannin and rapamycin on the IFN-γ-mediated induction of MCP-1 gene expression

The inhibitor LY294002 has been demonstrated previously to inhibit the kinase mammalian target of rapamycin (mTOR) independently of PI3K activity. The induction of iNOS was found to be inhibited by LY294002 and a dominant negative construct specifying for a mutant form of mTOR, but not by an alternative PI3K inhibitor, wortmannin (Kristof *et al.* 2003). In relation to the induction of MCP-1 expression by IFN-γ, LY294002 has been found to inhibit the response in endothelial cells, while wortmanin did not, and the effect of LY294002 was concluded to be independent of PI3K inhibition (Choi *et al.* 2004). The inhibitors wortmannin and rapamycin were therefore used in order to confirm that the IFN-γ-mediated induction of MCP-1 was dependent on PI3K and not mTOR. A second possibility is that mTOR acts as a downstream effector of PI3K in the regulation of MCP-1 expression. Previous studies have shown that mTOR is activated by PKB and that it may have a role in the activation of STAT1 (Bjournsti and Houghton 2004). Wortmannin is an antibiotic originally isolated in *Penicillium wortmannii* that irreversibly binds to the ATP binding pocket of the p110 subunit of PI3K (Arcaro and Wymann 1993). Rapamycin is an antibiotic produced by *Streptomyces hygroscopicus* that forms a complex with the FK506-binding protein (FKBP)-12 which then binds and prevents the activation of mTOR (Chen *et al.* 1995) (Table 2.2).

J774.2 cells were pretreated with LY294002 (5μM and 20μM), wortmannin (5μM and 20μM), rapamycin (200nM and 1μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which RNA was extracted. RT-PCR was carried out as before using primers specifying for murine MCP-1 and β-actin (Table 2.5). Figure 3.7 shows the PCR products resolved by agarose gel electrophoresis (Figure 3.7A) and band densities normalised to β-actin expression (Figure 3.7B). Wortmannin was found to inhibit IFN-γ-induced MCP-1 expression in a dose-dependent manner comparable to the inhibition with LY294002. Rapamycin however, reduced the stimulation of MCP-1 expression by a minimal degree in comparison with the effect of LY294002 or wortmannin, and only at a high concentration. The inhibition observed upon treatment with wortmannin supports the theory that activation of PI3K is necessary for the induction of MCP-1 by IFN-γ and that this is prevented by the action of LY294002.

A.





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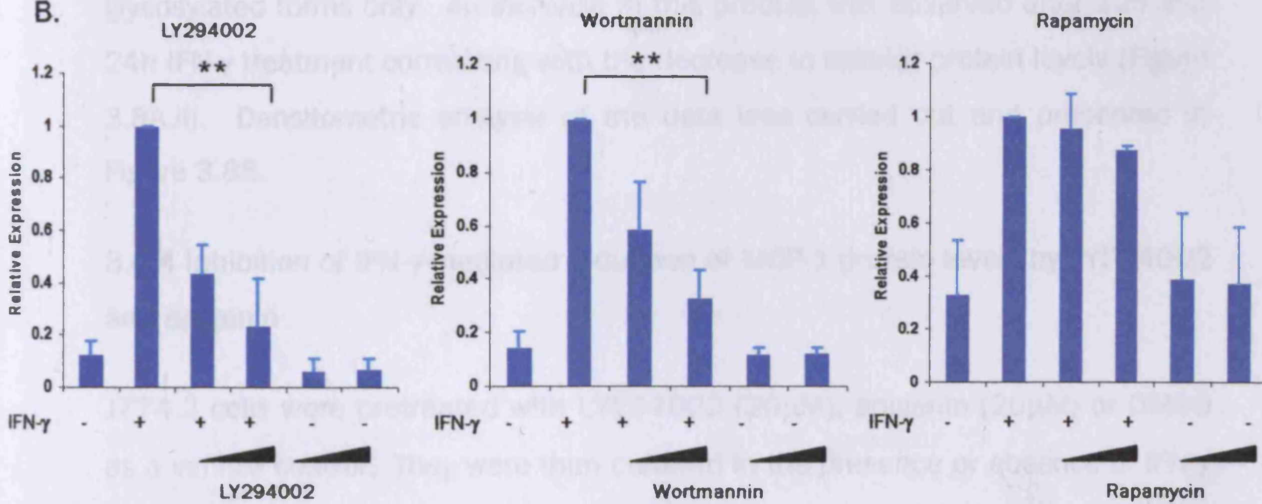


3.4.3 Induction of MCP-1 protein levels by IFN-γ

In order to confirm that the changes shown at the mRNA level for MCP-1 correspond to changes at the protein level, western blot analysis was carried out. Cells of the J774.2 cell line were stimulated with IFN-γ for periods of 0, 1, 3, 6, 12 and 24h after which whole cell protein extracts were prepared with buffers containing phosphatase inhibitors (Section 2.6.2). Western blot analysis was carried out on whole cell extracts (60μg) immunoblotting with a specific antibody raised against MCP-1 (Section 2.6.7-2.6.9) (Table 2.9). Bands for murine MCP-1 were seen in the range of 14-30kDa corresponding to various glycosylation products (Zhang *et al.* 1996). Cellular protein levels of MCP-1 were found to be maximal following 3h stimulation with IFN-γ and subsequently decreased (Figure 3.8A.i). Membranes were re-probed with anti-β-actin to ensure equal loading (Table 2.9). To determine whether this decrease in cellular protein was due to enhanced secretion of the MCP-1 protein, western blot analysis was performed on aliquots of extracellular growth medium collected over the same time course. Bands were seen in the range of 25-30kDa. The loss of the smaller sized products seen with cellular extracts is likely to be due to the secretion of fully glycosylated forms only. An increase in this product was observed after 12h and 24h IFN-γ treatment correlating with the decrease in cellular protein levels (Figure 3.8A.ii). Densitometric analysis of the data was carried out and presented in Figure 3.8B.

3.4.4 Inhibition of IFN-γ-mediated induction of MCP-1 protein levels by LY294002 and apigenin

J774.2 cells were pretreated with LY294002 (20μM), apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which phosphatase-free whole cell protein extracts were prepared. Western blot analysis was carried out on whole cell extracts as before, immunoblotting with specific antibodies raised against MCP-1 and β-actin. As shown in Figure 3.9, both LY294002 and apigenin attenuated the IFN-γ-mediated induction in MCP-1 protein levels. Densitometric analysis of the data was carried out and presented in Figure 3.9B.

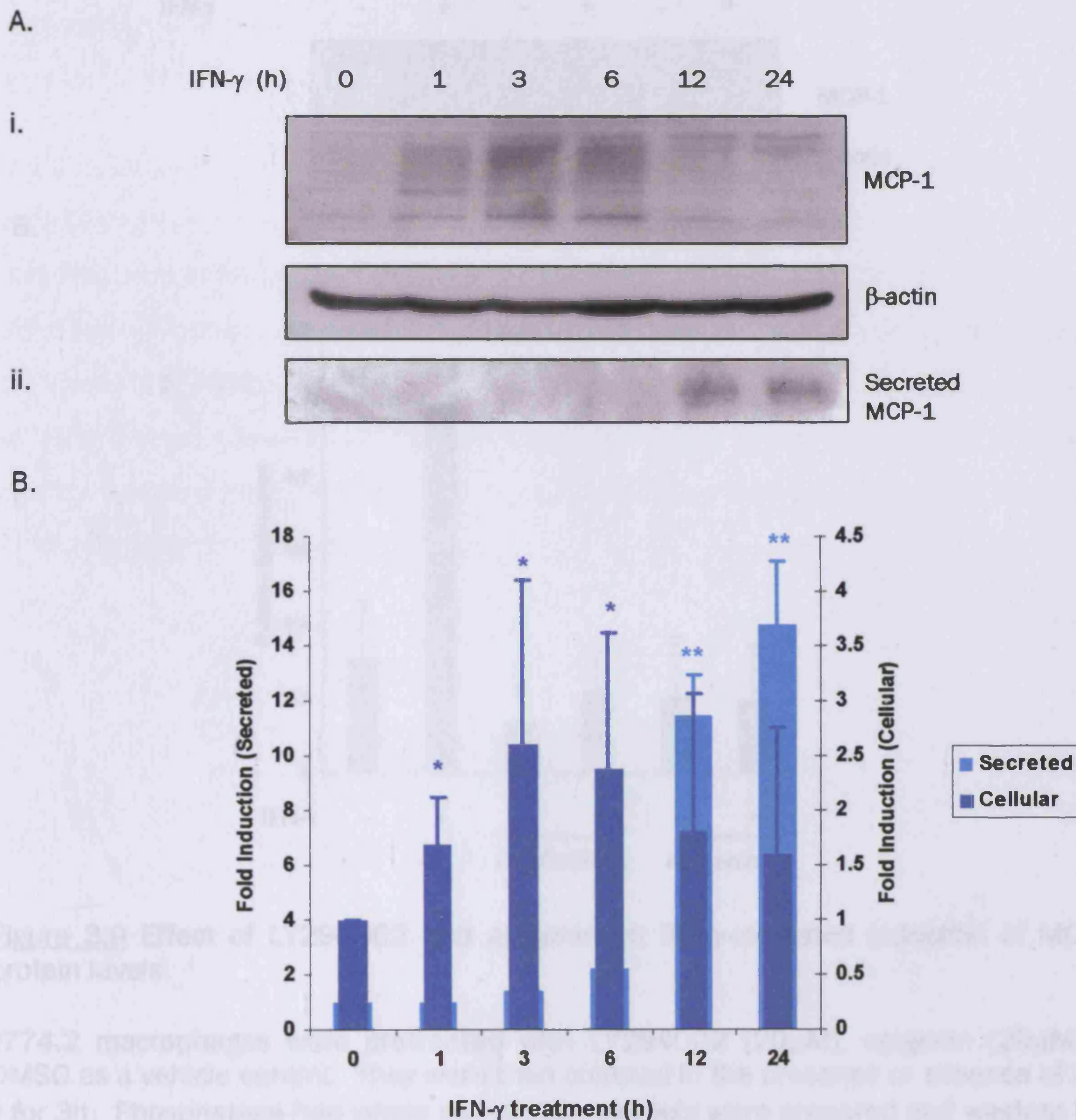


Figure 3.8 Induction of MCP-1 protein levels and secretion by IFN-γ

J774.2 cells were treated with IFN-γ for the times indicated and phosphatase-free whole cell protein extracts were prepared. Western blot analysis was carried out using antibodies specific for MCP-1 and β-actin as shown. Results are representative of four independent experimental series (A.i). Western blot analysis was also carried out on aliquots of growth medium obtained over the same time course. Results are representative of two independent experiments (A.ii). Densitometric analysis was carried out on the data and presented as the mean fold induction (±SD) as shown (B). *P<0.05; **P<0.01.

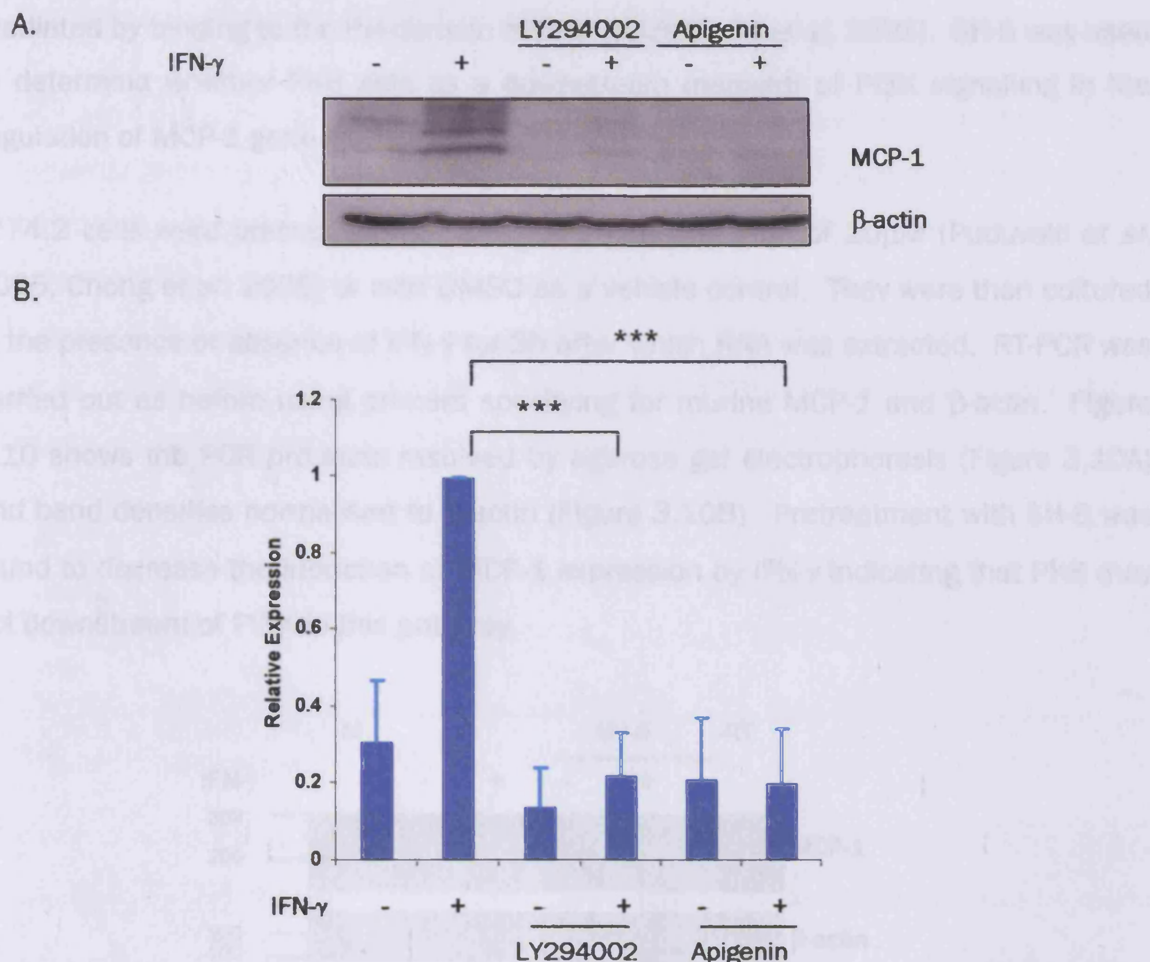


Figure 3.9 Effect of LY294002 and apigenin on IFN-γ-mediated induction of MCP-1 protein levels

J774.2 macrophages were pretreated with LY294002 (20μM), apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Phosphatase-free whole cell protein extracts were prepared and western blot analysis was carried out using an antibody specific for MCP-1 and β-actin (A). Densitometric analysis was carried out on the data from independent experiments (mean ±SD) (B). ***$P<0.001$

3.4.5 Inhibition of IFN-γ-mediated induction of MCP-1 gene expression by the pharmacological inhibitor of PKB activity SH-6

PKB is a well-characterised effector of PI3K signalling, downstream targets of which include GSK-3 and mTOR (Section 1.8.3). Previous studies have found that PKB is activated by IFN-γ in primary monocytes, THP-1 cells and fibroblasts (Navarro *et al.* 2003, Nguyen *et al.* 2001). The small molecule inhibitor SH-6 has been shown to be an effective and specific inhibitor of PKB activity, without altering the activation of the upstream kinase PDK-1. SH-6 is a phosphatidylinositol analog the action of which is

mediated by binding to the PH-domain of PKB (Kozikowski *et al.* 2003). SH-6 was used to determine whether PKB acts as a downstream mediator of PI3K signalling in the regulation of MCP-1 gene expression by IFN-γ.

J774.2 cells were pretreated with SH-6 at a concentration of 20μM (Puduvalli *et al.* 2005, Chong *et al.* 2005) or with DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which RNA was extracted. RT-PCR was carried out as before using primers specifying for murine MCP-1 and β-actin. Figure 3.10 shows the PCR products resolved by agarose gel electrophoresis (Figure 3.10A) and band densities normalised to β-actin (Figure 3.10B). Pretreatment with SH-6 was found to decrease the induction of MCP-1 expression by IFN-γ indicating that PKB may act downstream of PI3K in this pathway.

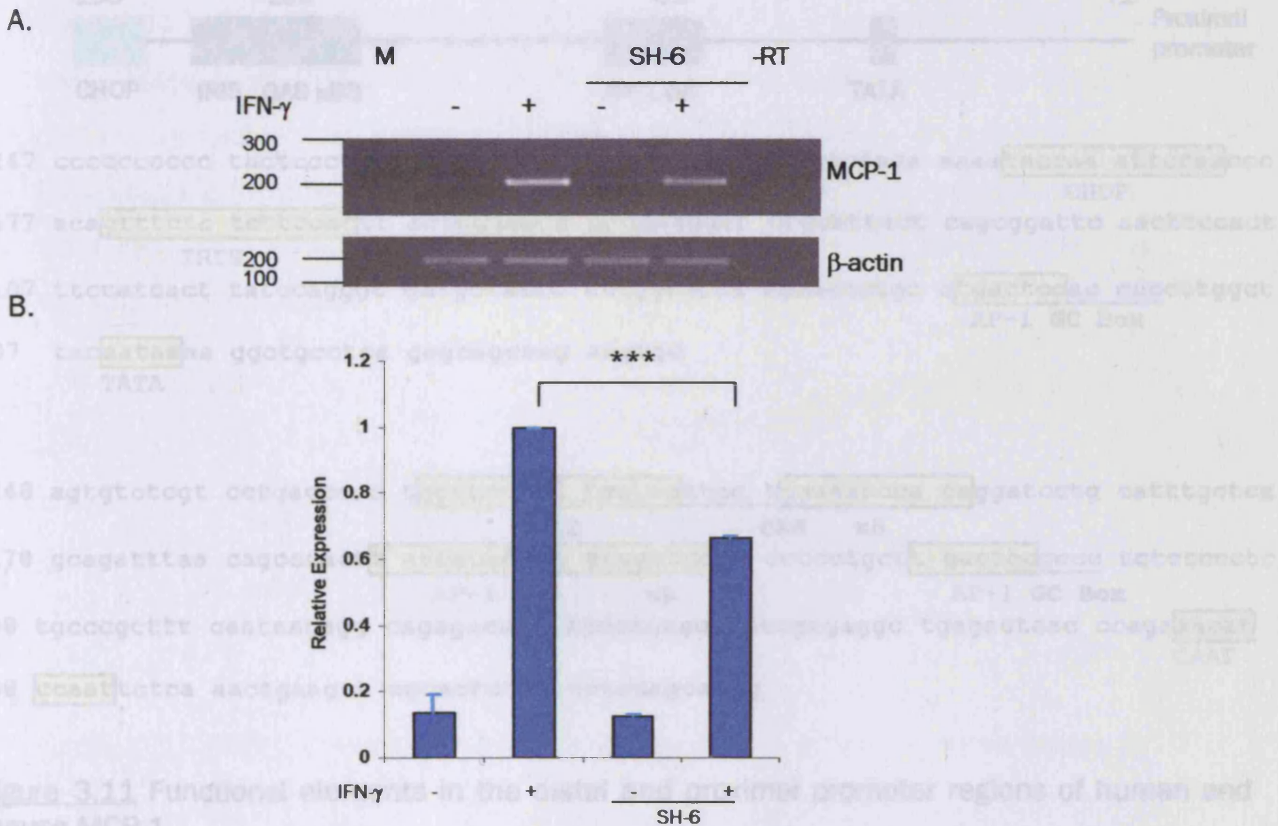


Figure 3.10 Inhibition of IFN-γ-mediated induction of MCP-1 expression by SH-6

J774.2 macrophages were pretreated with SH-6 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1 and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis from three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). ***P<0.001.

3.5 ACTIVATION OF THE MCP-1 PROMOTER BY IFN-γ

The expression of the MCP-1 gene is thought to be regulated by key distal and proximal promoter regions as discussed in Section 1.6.3. Figure 3.11 details the functional elements of the MCP-1 promoter. Previous studies in astrocytes have mapped IFN-γ-inducibility to a region of the proximal promoter of the human MCP-1 gene 213bp upstream of the transcriptional start site (Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998). The MCP-1[213]Luc construct contains this region, cloned into the pGL2-Basic luciferase reporter vector (Section 2.4.5).

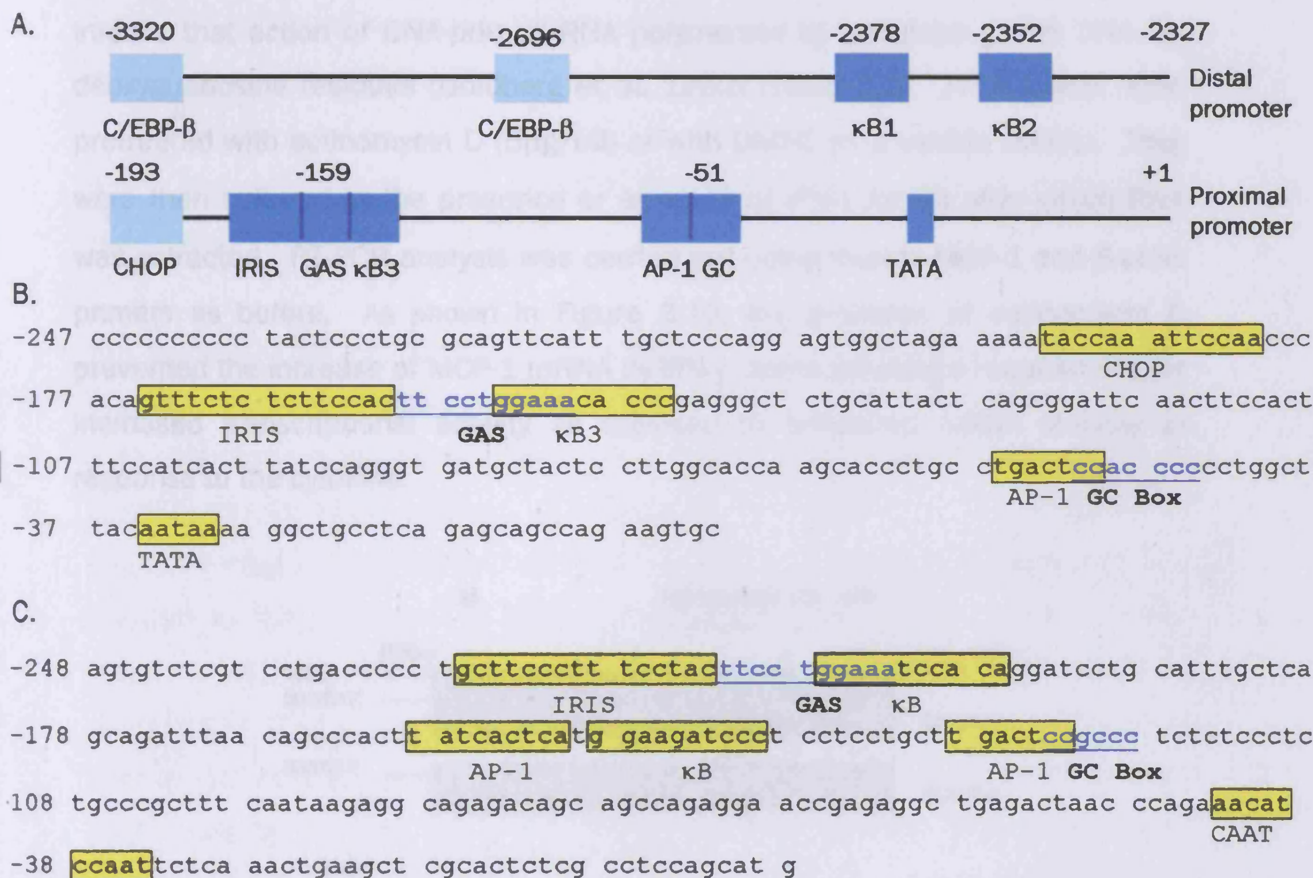


Figure 3.11 Functional elements in the distal and proximal promoter regions of human and mouse MCP-1

Schematic representation of the functional elements in the murine MCP-1 promoter (A). Sequence of the murine proximal MCP-1 promoter region (functional elements are highlighted) (adapted from Kodama *et al.* 2005, Lim *et al.* 2000, Valente *et al.* 1998, Zhou *et al.* 1998) (B). Sequence of the human proximal MCP-1 promoter region (functional elements are highlighted (adapted from Ueno *et al.* 2000, Valente *et al.* 1998, Ping *et al.* 1996) (C). Other functional C/EBP-binding elements may be present in the proximal promoter regions for which a specific site has not been determined (Abraham *et al.* 2005). Elements depicted in a lighter shade have been characterised in the rat MCP-1 promoter and the corresponding site in the mouse promoter highlighted.

3.5.1 IFN-γ-mediated upregulation of MCP-1 mRNA levels in macrophages is dependent on transcriptional activity but not mRNA stability

Although previous experiments in other cell types have demonstrated transcriptional activation of the MCP-1 promoter by IFN-γ (Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998), the chemical inhibitor of transcription, actinomycin D, was used to establish that IFN-γ increased the transcription of MCP-1 in the macrophage system and that the increase in mRNA levels was not mediated by a change in mRNA stability. Actinomycin D is an antibiotic that inhibits that action of DNA-primed RNA polymerase by complexing with DNA via deoxyguanosine residues (Goldberg *et al.* 1962) (Table 2.2). J774.2 cells were pretreated with actinomycin D (5μg/ml) or with DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which RNA was extracted. RT-PCR analysis was carried out using murine MCP-1 and β-actin primers as before. As shown in Figure 3.12, the presence of actinomycin D prevented the increase of MCP-1 mRNA by IFN-γ, demonstrating a requirement for increased transcriptional activity as opposed to enhanced mRNA stability in response to the cytokine.

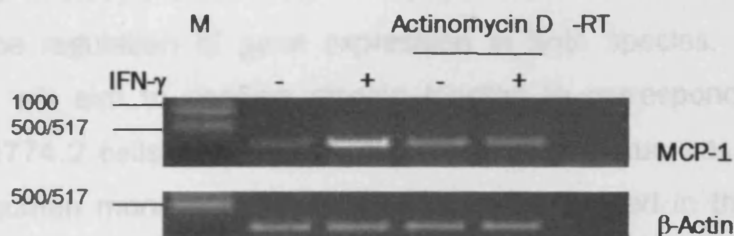


Figure 3.12 Effect of actinomycin D on the induction of MCP-1 mRNA levels by IFN-γ

J774.2 cells were pretreated with actinomycin D (5μg/ml) or with DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against MCP-1 and β-actin. The amplification products were analysed by agarose gel electrophoresis. The size of the DNA markers are indicated. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). Results shown are representative of two independent experiments.

3.5.2 Preparation of plasmid constructs for transfection based assays

Plasmid DNA was used to transform the bacterial *E. Coli* strain DH5α and extracted from a single colony by the Miniprep method (Section 2.5.9). Digestion with restriction endonucleases was carried out to establish that the identity of the plasmid was as expected (Section 2.5.11). Table 2.3 gives the restriction endonucleases used for the digestion of each plasmid and the fragment sizes expected. The same bacterial colony was subsequently used to prepare glycerol stocks and for further extraction of plasmid DNA by the Maxiprep method (Section 2.5.10). Dominant negative and luciferase reporter plasmid constructs are described in Section 2.4.5.

3.5.3 IFN-γ induces the activation of the MCP-1 promoter in a macrophage transfection system

The promoter construct MCP[213]Luc contains the human sequence for the MCP-1 promoter 213bp upstream of the transcriptional start site. As detailed in Figure 3.11, the proximal promoter of the murine MCP-1 gene is directly comparable and contains similar binding sites. MCP[213]Luc was therefore considered to be a suitable model to study the activation of the MCP-1 promoter in order to give data relevant to the regulation of gene expression in both species. Later studies (Section 4.4) will aim to confirm protein binding to corresponding regulatory elements in J774.2 cells. The transfection of MCP[213]Luc was carried out in cells of the human monocytic U937 cell line, differentiated in the presence of PMA. This system has been used successfully in previous transfection-based experiments in our laboratory for the study of macrophage gene expression (Irvine *et al.* 2005, Hughes *et al.* 2002). This approach also avoids difficulties, experienced within our laboratory and others, with the efficient transfection of J774.2 macrophages with exogenous DNA (Hughes *et al.* 2002, Hill *et al.* 1995).

U937 cells were transfected with MCP[213]Luc using the SuperFect™ Transfection method (Qiagen), following differentiation in the presence of PMA (Section 2.4.1). Transfected cells were treated with human IFN-γ for 12h (identified as optimal from a time-course analysis (data not shown)). Cells were

then harvested and extracts prepared for luciferase assay (Section 2.4.3-2.4.4). All transfections were carried out with samples in triplicate for at least three independent experiments. Results are presented as the mean fold induction (basal level assigned as 1) (±SD). A standard statistical t-test (Appendix IV) was performed on the data and changes in expression were found to be significant to the level indicated. Figure 3.13. shows an upregulation of MCP-1 promoter activity of approximately four fold in response to IFN-γ.

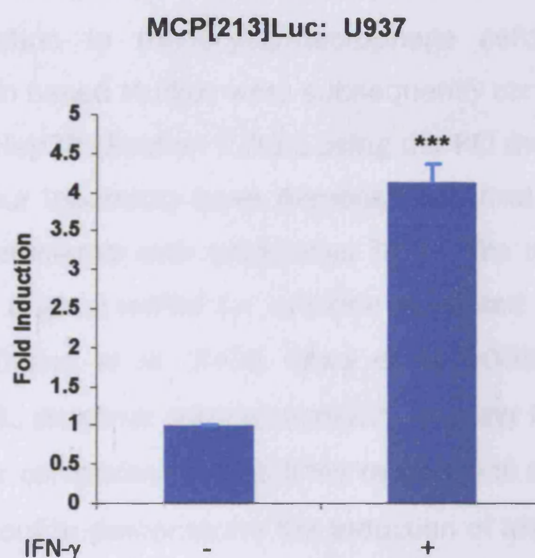


Figure 3.13 Stimulation of MCP-1 promoter activity by IFN-γ in macrophages

Cells were transfected with MCP[213]Luc and treated with IFN-γ for 12h. Relative counts were normalised to protein concentration and presented as the fold induction in response to IFN-γ, in relation to basal expression (assigned as 1). Results are presented as mean ±SD of three independent experiments. ***P<0.001.

3.5.4 Validation of a hepatocyte transfection system for studies of MCP-1 promoter activity

The transfection efficiency of U937 cells was sufficient to observe a reproducible induction in reporter gene expression in response to IFN-γ treatment (Figure 3.13). However, the efficiency of co-transfection assays utilising dominant negative expression plasmids was not adequate in this system to observe the effect of dominant negative mutants. Indeed, several laboratories have shown that macrophages cannot be transfected at high efficiency with exogenous DNA (Dokka *et al.* 2000, Kusumawati *et al.* 1999, Hill *et al.* 1995). Due to the difficulty of transfection in monocyte/macrophage cells with the required efficiency, transfection based studies were subsequently carried out in the human hepatocyte cell line, Hep3B (Section 2.3.1), using the PEI method (Section 2.4.2). Experiments within our laboratory have demonstrated that this cell line can be transfected at high efficiency with exogenous DNA. We have also shown that Hep3B cells provide a good model for cytokine regulated gene expression and responses to IFN-γ (Irvine *et al.* 2005, Foka *et al.* 2003, Irvine, S., personal communication, Ali, S., personal communication). In order to further validate the use of this system for comparison to the IFN-γ response in macrophages, RT-PCR analysis was carried out to demonstrate the induction of MCP-1 by IFN-γ, and the effect of LY294002 and apigenin on this, in Hep3B cells. The activation of the MCP-1 promoter by IFN-γ was also compared by transfection of the MCP[213]Luc reporter plasmid in U937 cells and Hep3B cells.

Hep3B cells were pre-treated with LY294002 (20μM), apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h after which RNA was extracted. RT-PCR was carried out as before using primers specifying for human MCP-1 and β-actin (Table 2.5). Figure 3.14A shows the PCR products resolved by agarose gel electrophoresis. Both LY294002 and apigenin attenuated the IFN-γ-mediated induction in MCP-1 mRNA levels.

Hep3B cells were transfected with MCP[213]Luc using the PEI method (Section 2.4.2). Transfected cells were treated with human IFN-γ for 6h (identified as

optimal from a time-course analysis (data not shown)). Cells were then harvested and extracts prepared for luciferase assay. Results are presented as the mean fold induction (basal level assigned as 1) (±SD). A standard statistical t-test was performed on the data and changes in expression were found to be significant to the level indicated. Figure 3.14B. shows an upregulation of MCP-1 promoter activity of approximately four-fold in response to IFN-γ, comparable to the level of induction in U937 cells.

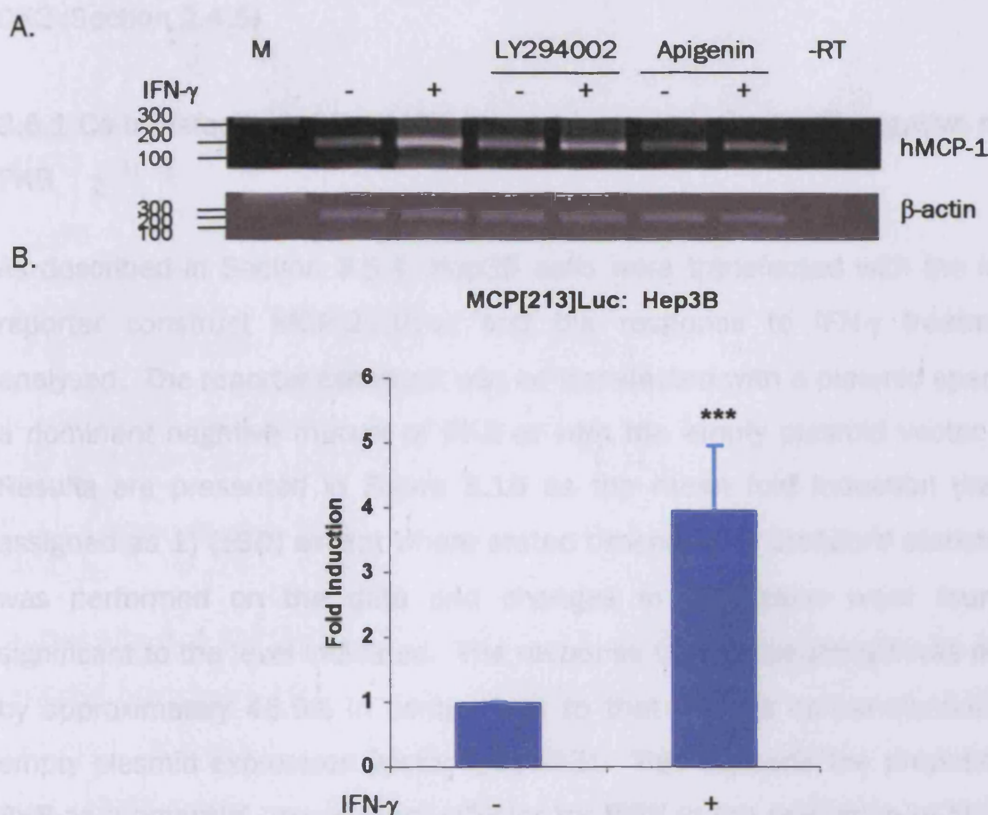


Figure 3.14 Stimulation of MCP-1 gene expression and promoter activity by IFN-γ in hepatocytes

Hep3B cells were pretreated with LY294002 (20μM), apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against hMCP-1 and β-actin. The amplification products were analysed by agarose gel electrophoresis. The size of the DNA markers are indicated. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). Results shown are representative of two independent experimental series (A). Cells were transfected with MCP[213]Luc and treated with IFN-γ for 6h. Relative counts were normalised to protein concentration and presented as the fold induction in response to IFN-γ, in relation to basal expression (assigned as 1). Results are presented as mean ±SD of six independent experiments (B). ***P<0.001.

3.6 EFFECT OF DOMINANT NEGATIVE INHIBITORS OF PKB AND CK2 ON THE ACTIVATION OF THE MCP-1 PROMOTER BY IFN-γ

The inhibitor studies detailed previously implicated CK2, PI3K and the downstream effector PKB in the induction of MCP-1 expression by IFN-γ. In order to confirm this role co-transfection experiments were performed using dominant negative inhibitor constructs for the downstream effector of PI3K, PKB, and for CK2 (Section 2.4.5)

3.6.1 Co-transfection of the MCP-1 promoter and a dominant negative mutant of PKB

As described in Section 3.5.4, Hep3B cells were transfected with the luciferase reporter construct MCP[213]Luc and the response to IFN-γ treatment (6h) analysed. The reporter construct was co-transfected with a plasmid specifying for a dominant negative mutant of PKB or with the empty plasmid vector pcDNA3. Results are presented in Figure 3.15 as the mean fold induction (basal level assigned as 1) (±SD) except where stated otherwise A standard statistical t-test was performed on the data and changes in expression were found to be significant to the level indicated. The response to IFN-γ treatment was decreased by approximately 46.9% in comparison to that in cells co-transfected with the empty plasmid expression vector (pcDNA3). This supports the proposed role of PKB as a potential downstream effector for PI3K in the regulation of MCP-1 gene expression by IFN-γ. The lack of a complete inhibition of the IFN-γ response suggests that additional pathways may also be involved in the mediation of this response that do not require PI3K/PKB.

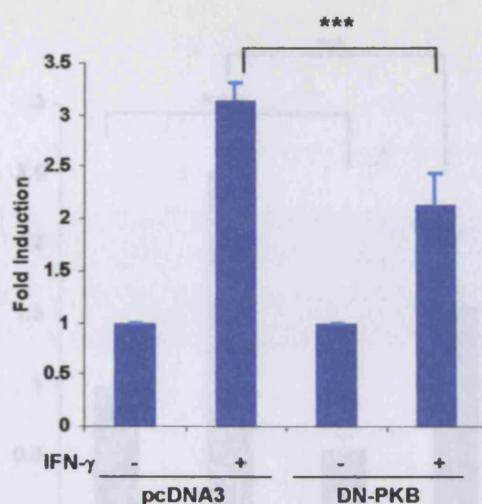


Figure 3.15 Effect of a dominant negative inhibitor of PKB on the stimulation of MCP-1 promoter activity by IFN-γ

Hep3B cells were transfected with MCP[213]Luc and a plasmid construct specifying for a dominant negative mutant form of PKB or the vector pcDNA3. Transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration and presented as the fold induction in response to IFN-γ, in relation to basal expression (assigned as 1). Results are presented as mean ±SD of five independent experiments. ***P<0.001.

3.6.2 Co-transfection of the MCP-1 promoter and a dominant negative mutant of CK2

As described in Section 3.5.4, Hep3B cells were transfected with the luciferase reporter construct MCP[213]Luc and the response to IFN-γ treatment (6h) analysed. The reporter construct was co-transfected with a plasmid specifying for a dominant negative mutant of CK2 (CK2αK68A) or with the empty plasmid vector pSG5. Co-transfection of dominant negative CK2 with MCP[213]Luc did not lead to a decrease in the relative fold induction in reporter gene expression upon stimulation with IFN-γ but caused a reduction in basal expression of approximately 45% in comparison with cells transfected with the empty expression vector (pSG5) only (Figure 3.16). Results are presented as the mean fold induction in comparison to the basal level for co-transfection with pSG5 (assigned as 1) (±SD). A standard statistical t-test was performed on the data and changes in promoter activity were found to be significant to the level indicated. This suggests that CK2 does have role in the regulation of MCP-1 expression but it is uncertain whether the kinase is involved in the regulation of constitutive promoter activity, as a mediator of IFN-γ-signalling, or possibly both.

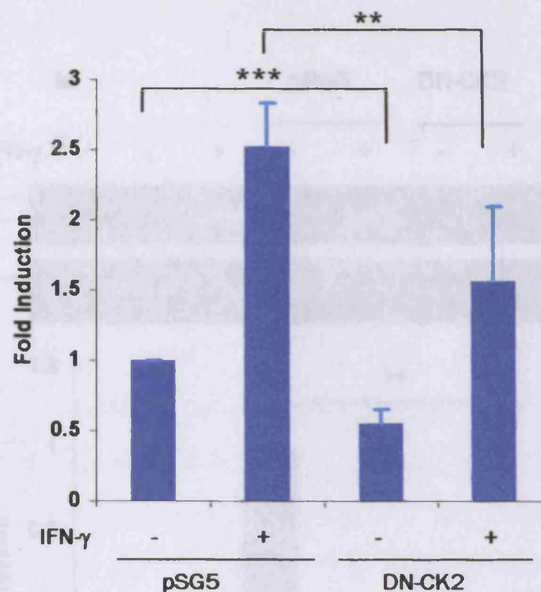


Figure 3.16 Effect of a dominant negative inhibitor of CK2 on the stimulation of MCP-1 promoter activity by IFN-γ

Hep3B cells were transfected with MCP[213]Luc and a plasmid construct specifying for a dominant negative mutant form of CK2 or the vector pSG5. Transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration. Results are presented as the fold induction with IFN-γ in relation to cells transfected with MCP[213]Luc and pSG5. Results are presented as mean ±SD of five independent experiments. ***$P<0.001$; **$P<0.01$.

3.6.3 RT-PCR analysis of the effect of dominant negative CK2 on the induction of MCP-1 gene expression by IFN-γ

In order to further address the role of CK2 in the induction of MCP-1 expression by IFN-γ, the effect of dominant negative inhibition of CK2 on endogenous MCP-1 mRNA expression in Hep3B cells was also assessed. Hep3B cells were transfected by the PEI method with a plasmid encoding a dominant negative mutant of CK2 or the empty plasmid vector pSG5. Cells were then stimulated with human IFN-γ for 3h after which RNA was extracted. RT-PCR was carried out using primers specific for human MCP-1 and β-actin as before. The products were resolved by agarose gel electrophoresis (Figure 3.17A) and band densities normalised to β-actin (Figure 3.17B). Dominant negative CK2 inhibited the induction of MCP-1, confirming the effect of apigenin.

A.

B.

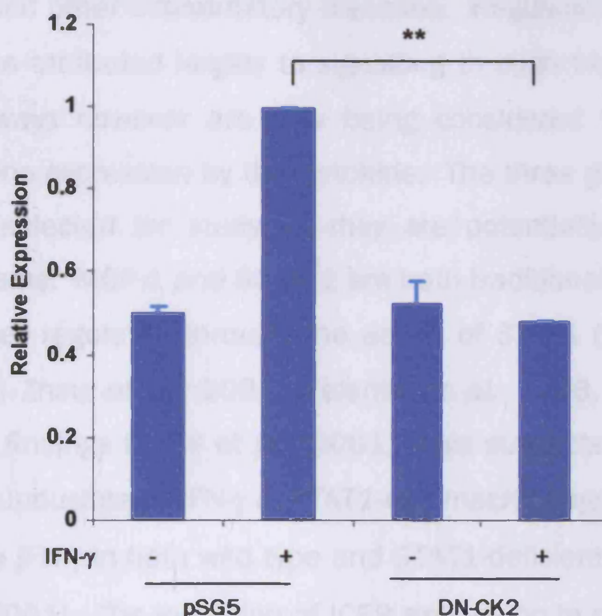


Figure 3.17 Effect of a dominant negative inhibitor of CK2 on the induction of MCP-1 mRNA expression by IFN-γ

Hep3B cells were transfected with a plasmid specifying for a dominant negative mutant form of CK2 or the vector pSG5. Transfected cells were incubated in the presence or absence of human IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against hMCP-1 and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using untreated RNA). Results are representative of three independent experiments. **$P<0.01$.

3.7 DISCUSSION

Macrophages have an important role in the progression of atherosclerosis (Section 1.4.4). The inflammatory cytokine IFN-γ regulates the expression of numerous genes in these cells and studies of the mechanisms involved in such responses may provide potential therapeutic targets for the treatment of atherosclerosis and other inflammatory diseases. Regulation of gene expression by IFN-γ has been attributed largely to signalling through the JAK-STAT pathway. Alternative pathways however are now being considered to contribute to the modulation of gene expression by this cytokine. The three genes MCP-1, SOCS-1 and ICER were selected for study as they are potentially regulated by three distinct mechanisms. MCP-1 and SOCS-1 are both traditionally recognised as IFN-γ-stimulated genes regulated through the action of STAT1 (Cornish *et al.* 2003, Alexander 2002, Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998). However, recent findings by Gil et al. (2001) have suggested that while SOCS-1 expression is not inducible by IFN-γ in STAT1-null macrophages, MCP-1 expression is upregulated by IFN-γ in both wild type and STAT1-deficient cells (Ramana *et al.* 2002, Gil *et al.* 2001). The induction of ICER expression in response to IFN-γ has been studied previously in our laboratory and represents a potentially novel CK2-dependent mechanism of gene regulation (Mead *et al.* 2003).

RT-PCR analysis of the time-dependent induction of MCP-1, SOCS-1 and ICER in J774.2 macrophages found the expression of all three genes to be maximally induced at a 3h time point (Figure 3.2). While MCP-1 and SOCS-1 expression remained at the same level for the duration of the time course, levels of ICER mRNA were reduced at subsequent time points suggesting that further signalling events are induced that inhibit ICER expression. ICER itself is likely to be responsible for this inhibition in a negative feedback mechanism, or other genes regulated in response to IFN-γ may act to suppress the transcription of ICER. It is clear from Figure 3.2 that the ICER I transcript is preferentially expressed in response to IFN- γ in macrophages.

3.7.1 Role of CK2 in the regulation of macrophage gene expression by IFN-γ

The protein kinase CK2 has been found in our laboratory to be involved in the suppression of LPL gene transcription and in the induction of ICER expression by IFN-γ through potentially novel pathways (Mead *et al.* 2003, Hughes *et al.* 2002). Previous work in our laboratory has also shown that IFN-γ induces CK2 activity in macrophages, and that this is inhibited, as expected, by the CK2 inhibitor, apigenin. Maximal activation of CK2 was observed following 2h stimulation with IFN-γ that would be consistent with a role for this kinase in the regulation of the genes selected, that are maximally induced after 3h cytokine treatment (Mead *et al.* 2003). Apigenin was used to determine whether CK2 has a role in the IFN-γ induced regulation of MCP-1 and SOCS-1 in macrophages.

Pretreatment of IFN-γ stimulated cells with apigenin resulted in the inhibition of IFN-γ induced upregulation of gene expression for all the genes studied (Figure 3.4). While implicating CK2 in the regulation of these genes, these findings also raise the possibility of a global role for CK2 in the IFN-γ-mediated modulation of macrophage gene expression. The SOCS-1 gene is known to be regulated through JAK-STAT signalling (Cornish *et al.* 2003) while ICER and MCP-1 have been suggested to be regulated by independent pathways (Mead *et al.* 2003, Gil *et al.* 2001). A CK2-dependent pathway may prove to be independent of JAK-STAT signalling, with both pathways being involved in the regulation of certain genes, or it may be a novel component of the JAK-STAT signalling pathway. This was a subject that was to be investigated further during the course of this study.

3.7.2 Selective role of PI3K in the regulation of MCP-1 by IFN-γ

The inhibitor LY294002 has been shown in our laboratory to prevent the IFN-γ-mediated suppression of LPL gene expression in macrophages (also dependent on CK2 activity), implicating PI3K in the IFN-γ-mediated regulation of this gene (Tengku-Muhammad *et al.* 1999b, Evans, S., personal communication). A number of other studies have also demonstrated dependence of IFN-γ signalling pathways on the activation of PI3K as discussed in Section 1.9. The effect of

PI3K inhibition by LY294002 on the IFN-γ-mediated induction of MCP-1, SOCS-1 and ICER was therefore investigated.

Initial work on the regulation of ICER was carried out in relation to a potential involvement in the suppresion of LPL gene expression in macrophages by IFN-γ. Transfection of cells with a plasmid construct for ICER was found to result in reduced LPL expression (Mead 2002). Possibly ICER represses the transcription of Sp3 (that regulates LPL promoter activity), so accounting for the reduced levels of this protein observed following stimulation with IFN-γ (Hughes *et al.* 2002). If ICER is acting upstream in the regulation of LPL it is conceivable that the role of CK2 is in the upregulation of ICER expression and that PI3K is necessary for this. However, the IFN-γ-mediated induction in ICER expression was not affected by the inhibitor LY294002, indicating that PI3K is not involved in its regulation (Figure 3.5). This does not rule out the possibility that ICER is involved in the suppression of LPL, with a dual role for CK2, in the PI3K-independent upregulation of ICER and subsequently the PI3K-dependent downregulation of LPL. Similarly, LY294002 did not affect IFN-γ-mediated induction of SOCS-1 expression (Figure 3.5). However MCP-1 expression following stimulation with IFN-γ for 3h was reduced by LY294002 in a concentration dependent manner (Figure 3.5). PI3K therefore, in addition to CK2, appears to be selectively involved in the IFN-γ-mediated regulation of this gene. A study by Bian *et al* (2004). has previously demonstrated a similarly selective role for PI3K in the the regulation of MCP-1 expression in response to the cytokines IL-1β and TNF-α. In this case LY294002 was found to attenuate the induction of MCP-1 gene expression by the cytokines but not that of IL-8 (Bian *et al.* 2004). Hwang *et al.* (2004) also showed that PI3K was required for the induction of iNOS mRNA expression in microglial cells through the activation of STAT1 but that IFN-γ-stimulated expression of IRF-1 and IP-10 was unaffected by the presence of the inhibitor.

Activation of PI3K in response to IFN-γ has been suggested to function in both JAK-STAT-dependent and -independent pathways (Section 1.9). It is tempting to speculate that the differential dependence of the regulation of MCP-1 and SOCS-1 expression by IFN-γ on PI3K is linked to the differential dependence on STAT1

shown by Gil *et al.* (2001). In order to further investigate the role of PI3K in IFN-γ-signalling, the regulation of MCP-1 expression by the cytokine was studied in more detail.

The inhibition of MCP-1 gene expression by LY294002 and apigenin, as seen in macrophages of the J774.2 cell line, was similarly demonstrated in primary human monocyte-derived macrophages, indicating the relevance of these studies to conditions *in vivo* and ruling out cell line-specific effects (Figure 3.6). The induction of MCP-1 protein expression by IFN-γ and the effect of these inhibitors on this response was also determined by western blot analysis. IFN-γ was found to mediate a similar effect on the level of MCP-1 protein within the cell, maximal levels being reached following 3h stimulation with the cytokine. A decrease in cellular protein at subsequent time points correlated with increased MCP-1 secretion (Figure 3.8). The inhibitors LY294002 and apigenin also prevented the stimulation of MCP-1 protein expression by IFN-γ indicating that the observations of MCP-1 expression at the mRNA level are also relevant at the protein level (Figure 3.9).

In order to confirm that the inhibitory effect of LY294002 was specific to the inhibition of PI3K, a second inhibitor, wortmannin was utilised. As LY294002 has also been reported to inhibit mTOR and because this molecule has been implicated as a downstream effector of PI3K in the regulation of STAT1, the effect of treatment with the mTOR inhibitor, rapamycin, on the response of MCP-1 expression to IFN-γ was also analysed. Wortmannin was found to inhibit the IFN-γ response in a similar manner to LY294002 while rapamycin only had a minimal effect at high dose (Figure 3.7). This suggests that LY294002 is affecting the IFN-γ induced expression of MCP-1 through the inhibition of PI3K and that mTOR is unlikely to be acting as a downstream effector in this signalling pathway.

Previous experiments have demonstrated transcriptional activation of the MCP-1 promoter by IFN-γ in astrocytes and osteoblasts but to our knowledge conclusive studies have not been carried out in macrophages (Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998). The chemical inhibitor of transcription, actinomycin

D, was used to confirm that IFN-γ increased the transcription of MCP-1 in the macrophage system and that the response is not mediated by an increase in mRNA stability. The expression of the MCP-1 gene is thought to be regulated by key distal and proximal promoter regions as discussed in Section 1.6.3. Functional elements in the proximal MCP-1 promoter include putative sites for Sp1, STAT1, AP-1, NF-κB and C/EBP binding (Figure 3.11). Previous studies, in other cell lines, have mapped IFN-γ-inducibility to a region of the proximal promoter of human MCP-1 213bp upstream of the transcriptional start site (Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998).

A luciferase reporter construct containing this promoter region (MCP[213]Luc) was used to show this response in macrophages. As this construct contains the human sequence for the MCP-1 promoter, and to avoid difficulties associated with the transfection of J774.2 macrophages, transfections were carried out in human cells. U937 monocytes differentiated in the presence of PMA have been successfully used in previous transfection-based experiments in our laboratory (Hughes *et al.* 2002, Irvine *et al.* 2005). Activation of reporter expression from MCP[213]Luc in U937 cells was demonstrated in response to stimulation with IFN-γ. However, the efficiency of co-transfection assays utilising dominant negative plasmids was not adequate in this system to observe the dominant negative effect. Due to the difficulty of transfection in monocyte/macrophage cells with the required efficiency, transfection based studies were subsequently carried out in the human hepatocyte cell line, Hep3B, using the PEI method. In order to validate the use of this system, RT-PCR analysis was carried out demonstrating the induction of MCP-1 expression by IFN-γ and the inhibition of this response by LY294002 and apigenin. The IFN-γ-mediated activation of MCP[213]Luc was also compared in U937 cells and Hep3B cells and found to be of a similar level (Figure 3.13 and 3.14).

A number of signalling effectors function downstream of PI3K (Figure 1.3). PKB has been well characterised as a mediator of PI3K signalling and its activity has been shown in various studies to be inducible by IFN-γ (Navarro *et al.* 2003, Nguyen *et al.* 2001). Data from the laboratory of Nguyen *et al.* (2001) has also

implicated PKB in the phosphorylation of STAT1 on Serine727. The IFN-γ-mediated induction of MCP-1 expression was found, by RT-PCR analysis, to be attenuated by the small molecule inhibitor of PKB activity, SH-6 (Figure 3.10). Co-transfection of a dominant negative construct specifying for a mutant form of PKB with MCP[213]Luc inhibited the induction by IFN-γ by approximately 46.9% (Figure 3.15). Together these results implicate PKB as a likely downstream effector of PI3K in the IFN-γ-mediated regulation of MCP-1 gene expression. A previous study by Murao *et al.* (2000), that investigated the role of PI3K and PKB in the induction of MCP-1 expression by TNF-α in ECs, also found that a constitutively active form of PKB was capable of mediating an increase in MCP-1 expression, providing further support for the role of PKB in the activation of the MCP-1 promoter (Murao *et al.* 2000).

3.7.3 Role of CK2 in the induction of MCP-1 expression by IFN-γ

The role of CK2 in the regulation of MCP-1 expression by IFN-γ, identified using the inhibitor apigenin, was supported by transfection of a dominant negative form of the kinase (CK2αK68A) in Hep3B cells. The induction of MCP-1 mRNA expression by IFN-γ was inhibited in cells transfected with the dominant negative mutant compared to transfection with the empty vector (pSG5). The effect of apigenin on IFN-γ-mediated regulation of MCP-1 expression was also demonstrated in human primary macrophages.

Co-transfection of dominant negative CK2 with MCP[213]Luc resulted in a decrease in both the basal and IFN-γ-induced reporter gene expression. This observation suggests the possibility that CK2 may function in the constitutive expression of MCP-1 and potentially the other genes studied. A role for CK2 in constitutive gene expression would be fitting with the known properties of the kinase. CK2 is unusual in that it is a constitutively active kinase, although the activity has been shown by work in our laboratory to be increased by IFN-γ treatment (Mead *et al.* 2003). Other laboratories have also shown an induction in CK2 activity in certain contexts (Peppercock *et al.* 1991, Sayed *et al.* 2000, Brenneisen *et al.* 2002). It is possible therefore, that CK2 may have dual roles in

both the constitutive expression of the genes studied and in the IFN-γ response. CK2 has been shown to be involved in regulating the transcriptional activity of RNA polymerase III, responsible for the expression of tRNA and small ribosomal RNAs, and in the regulation of transcription of mRNA transcripts by RNA polymerase II (Cabrejos *et al.* 2004, Llorens *et al.* 2003, Hu *et al.* 2003, Johnston *et al.* 2002).

The low basal level of mRNA expression for the genes studied makes the function of CK2 in the regulation of constitutive promoter activity difficult to ascertain from RT-PCR experiments. It is possible that the effect of dominant negative CK2 on the basal activity of the MCP[213]Luc promoter may be due to the non-chromatin context of the promoter or alternatively, a property of the cellular system, with a differential response between Hep3B hepatocytes and J774.2 macrophages. Further studies of the function of CK2 in the regulation of MCP-1 gene expression may help to establish this. It also remains to be determined whether PI3K and CK2 are functioning in a linear signalling pathway or in two distinct regulatory systems.

CHAPTER FOUR:

MECHANISMS IN THE REGULATION OF MCP-1 GENE EXPRESSION BY IFN-γ

CHAPTER 4. MECHANISMS IN THE REGULATION OF MCP-1 GENE EXPRESSION BY IFN-γ

4.1 INTRODUCTION

As discussed in Section 1.6, MCP-1 has an important role in the progression of atherosclerosis. Knock-out of the MCP-1 gene, or its receptor CCR2, in apoE- and LDL-R-deficient mice dramatically inhibits lesion development (Dawson *et al.* 1999, Gosling *et al.* 1999, Boring *et al.* 1998, Gu *et al.* 1998). The regulation of this chemokine by inflammatory mediators is therefore of particular therapeutic interest. Further investigations of the mechanisms involved in the regulation of MCP-1 gene expression by IFN-γ were carried out with the aim of elucidating a novel pathway involved in IFN-γ-induced signalling and identifying potential targets for pharmacological inhibiton of MCP-1 function in atherosclerosis.

4.1.1 JAK-STAT signalling in the regulation of MCP-1 expression by IFN-γ

Previous studies have implicated STAT1 in the regulation of MCP-1 expression in response to IFN-γ stimulation. A GAS site present in the proximal promoter of the human MCP-1 gene has been found to be necessary for the stimulation of promoter activity by IFN-γ in astrocytes (Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998). However the analysis of STAT1-knock out BMMs in an independent study by Gil *et al.* (2001), suggested that STAT1 is not essential for the upregulation of MCP-1 by IFN-γ and that a STAT1-independent signalling mechanism may have a role. In this study, the presence of JAK1 in addition to IFN-γR was required for the induction of other genes regulated independently of STAT1.

The kinases PI3K (and the downstream effector PKB) and CK2 were shown in Chapter 3 to be necessary for the maximal induction of MCP-1 by IFN-γ. These kinases may potentially be involved in either STAT1-dependent or -independent mechanisms. Work in other laboratories has linked PI3K to the JAK-STAT pathway (Section 1.9.4) although the activation of PI3K by IFN-γ has also been observed in

JAK1- and JAK2-deficient cells (Nguyen *et al.* 2001). Our laboratory has shown that CK2 activity is induced by IFN-γ and that this is not inhibited by the JAK2 inhibitor AG490 (Mead *et al.* 2003, Evans, S., personal communication). This is consistent with our previous finding that the specific JAK2 inhibitor, AG490, does not suppress the stimulation of ICER gene expression by IFN-γ, in contrast to the effect of the CK2 inhibitor apigenin (Mead *et al.* 2003).

4.2.2 Experimental strategy

Work in this chapter was carried out with the aim of determining the role of JAK-STAT signalling in the regulation of MCP-1 gene expression by IFN-γ and the function of CK2 and PI3K either in relation to the JAK-STAT pathway or an independent mechanism. This was to be achieved through the continuing use of pharmacological inhibitors and dominant negative mutants in order to establish a requirement for certain mediators, in particular, the components of the JAK-STAT pathway JAK1, JAK2 and STAT1.

The function of CK2 and PI3K in the IFN-γ response was studied by analysing changes in the activity of these kinases in response to cytokine treatment. An IFN-γ-mediated induction in CK2 activity has been demonstrated previously in the laboratory (Mead *et al.* 2003, Evans, S., personal communication) so the data presented concentrates on changes in the activation of the downstream effector of PI3K, PKB. The potential requirement for these kinases in the regulation of STAT1 activity was determined by assessing the effect of pharmacological inhibitors and dominant negative mutants for CK2 and PKB on IFN-γ-mediated phosphorylation of STAT1 and the activation of a STAT1-regulated promoter.

Further studies of the factors required for the activation of the MCP-1 promoter were to be carried out by EMSA and ChIP analyses of promoter binding combined with the use of dominant negative mutants. Co-immunoprecipitation was also employed in order to study the interaction of particular mediators both constitutively and in response to IFN-γ treatment. Figure 4.1 illustrates the overall experimental strategy for the work covered in this chapter.

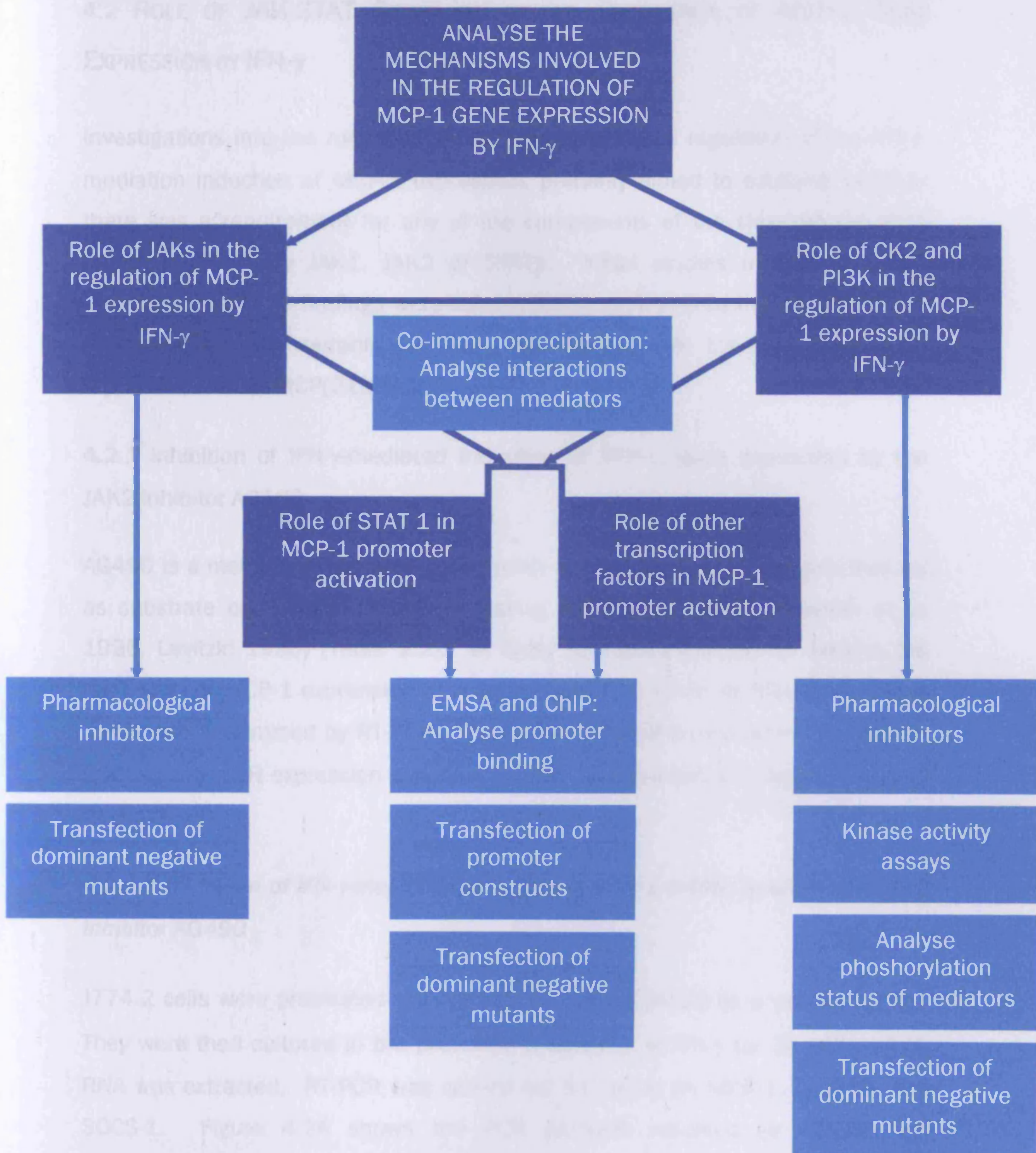


Figure 4.1 Experimental strategy

Further analysis of the signalling mechanisms involved in the induction of MCP-1 gene expression by IFN-γ will focus on determining the role of the JAK-STAT pathway, CK2 and PI3K in this response. Aims were to be achieved with the continuing use of pharmacological inhibitors and dominant negative mutants combined with analyses of alterations in kinase activity, promoter binding and the interactions between various mediators.

4.2 Role of JAK-STAT Signalling in the Regulation of MCP-1 Gene Expression by IFN-γ

Investigations into the role of JAK-STAT signalling in the regulation of the IFN-γ-mediation induction of MCP-1 expression, primarily aimed to establish whether there was a requirement for any of the components of the classical JAK-STAT pathway, principally JAK1, JAK2 or STAT1. Initial studies utilised the JAK2 inhibitor AG490 and findings were subsequently confirmed by the transfection of dominant negative mutants for these components with the MCP-1 promoter reporter construct MCP[213]Luc.

4.2.1 Inhibition of IFN-γ-mediated induction of MCP-1 gene expression by the JAK2 inhibitor AG490

AG490 is a member of the tyrphostin family of tyrosine kinase inhibitors that act as substrate competitors without affecting the binding of ATP (Meydan *et al.* 1996, Levitzki 1990) (Table 2.2). In order to assess the role of JAKs in the regulation of MCP-1 expression in macrophages, the effect of AG490 on mRNA levels was determined by RT-PCR. The effect on the IFN-γ-mediated induction of SOCS-1 and ICER expression was also studied as a positive and negative control respectively.

4.2.1.1 Inhibition of IFN-γ-mediated induction of MCP-1 mRNA levels by the JAK2 inhibitor AG490

J774.2 cells were pretreated with AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h, after which RNA was extracted. RT-PCR was carried out as before for MCP-1, ICER I/II and SOCS-1. Figure 4.2A shows the PCR products resolved by agarose gel electrophoresis and band densities normalised to β-actin (Figure 4.2B). For ICER I/II the level of mRNA did not differ between IFN-γ stimulated samples and those treated with AG490 in addition to IFN-γ, suggesting that JAK2 is not involved in the regulation of ICER expression. IFN-γ-mediated induction of SOCS-1 expression was reduced by AG490 as expected. MCP-1 expression was also inhibited by

pretreatment with AG490 so implicating JAK2 in the regulation of this gene by IFN-γ.

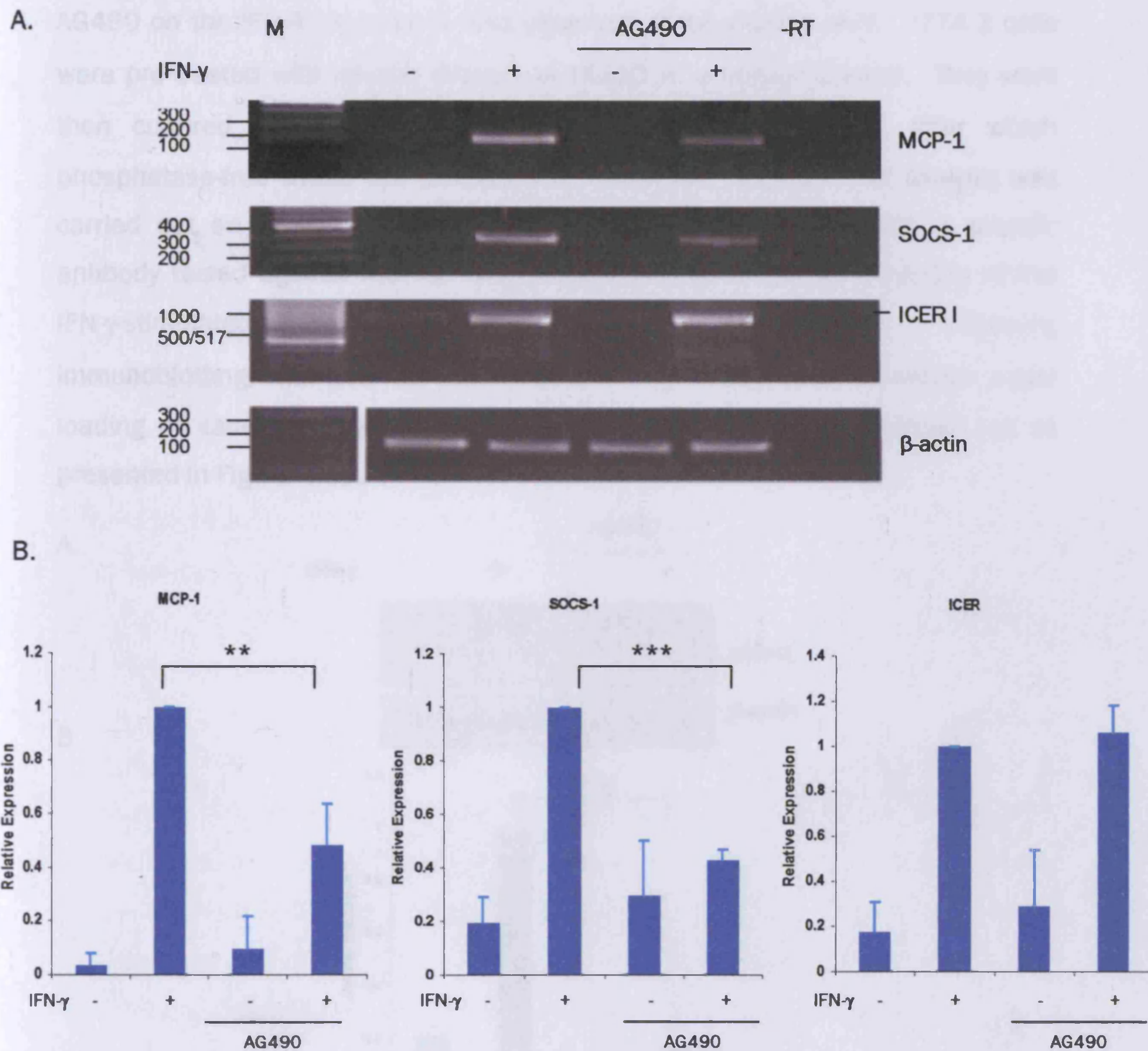


Figure 4.2 Inhibition of IFN-γ-mediated induction of MCP-1 mRNA expression by AG490

J774.2 macrophages were pretreated with AG490 (50μM) or DMSO as a vehicle control. They were then incubated in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against: MCP-1; SOCS-1; ICER I; β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The sizes of DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the experssion of β-actin as determined by densitometric analysis from four independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). **P<0.01; ***P<0.001.

4.2.1.2 Inhibition of IFN-γ-mediated induction of MCP-1 protein levels by the JAK2 inhibitor AG490

Western blot analysis was also carried out in order to confirm that the effect of AG490 on the IFN-γ-response is also observed at the protein level. J774.2 cells were pre-treated with AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h, after which phosphatase-free whole cell extracts were prepared. Western blot analysis was carried out on whole cell extracts as before, immunoblotting with a specific antibody raised against MCP-1. The results showed a marked inhibition of the IFN-γ-stimulated induction of MCP-1 expression by AG490. Following immunoblotting, membranes were re-probed with anti-β-actin to ensure equal loading of samples (Figure 4.3A). Densitometric analysis was carried out as presented in Figure 4.3B.

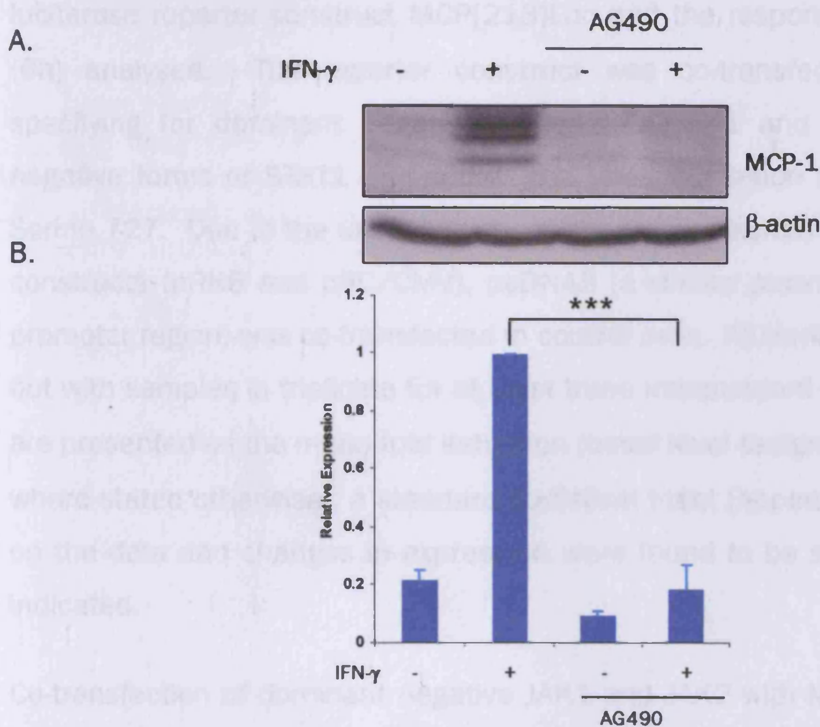


Figure 4.3 Inhibition of IFN-γ-mediated induction of MCP-1 protein levels by AG490

J774.2 cells were pre-treated with AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Phosphatase-free whole cell protein extracts were prepared and western blot analysis was carried out using antibodies specific for MCP-1 and β-actin (A). Densitometric analysis was carried out on the data (mean ±SD) from three independent experiments (B). ***P<0.001.

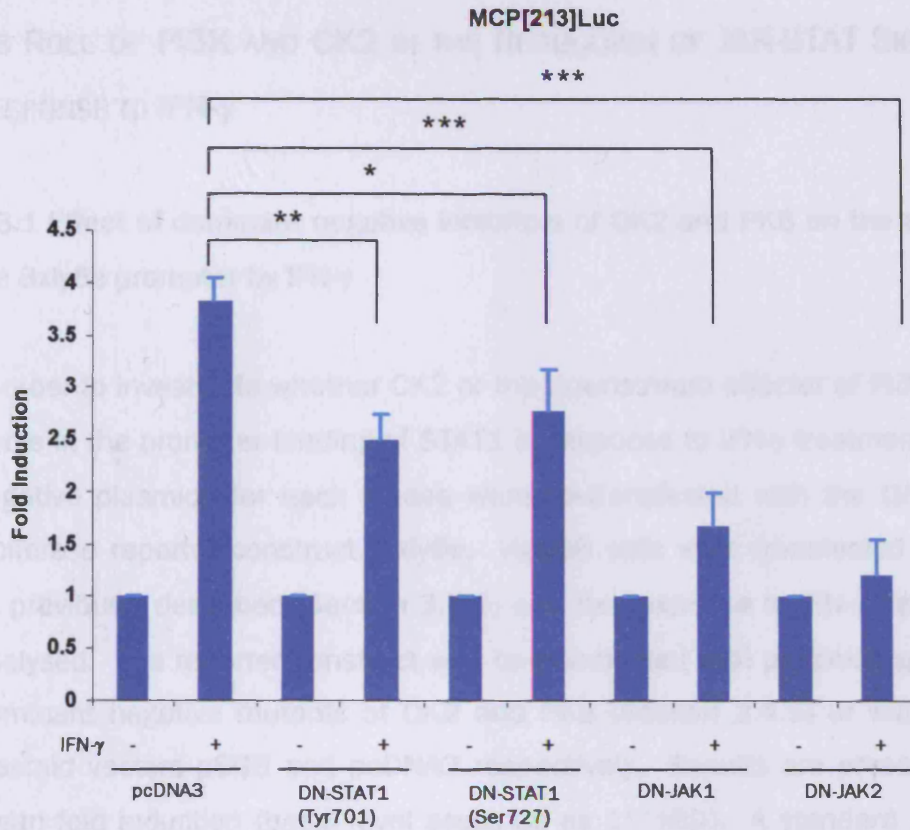
4.2.2 Effect of dominant negative inhibitors of JAK1, JAK2 and STAT1 on the activation of the MCP-1 promoter by IFN-γ

The luciferase reporter construct MCP[213]Luc, containing a 213bp region of the proximal human MCP-1 promoter, was previously shown to be responsive to IFN-γ stimulation in macrophages and in a Hep3B transfection system (Section 3.5). In order to confirm the role of JAKs in the regulation of MCP-1 gene expression by IFN-γ and to ascertain whether there is a requirement for STAT1, dominant negative constructs specifying for inactive mutants of JAK1, JAK2 and STAT1 (Section 2.4.5) were co-transfected with MCP[213]Luc. Co-transfections were also carried out using the GAS-regulated reporter plasmid, 3xly6e, as a positive control for the action of the dominant negative inhibitors.

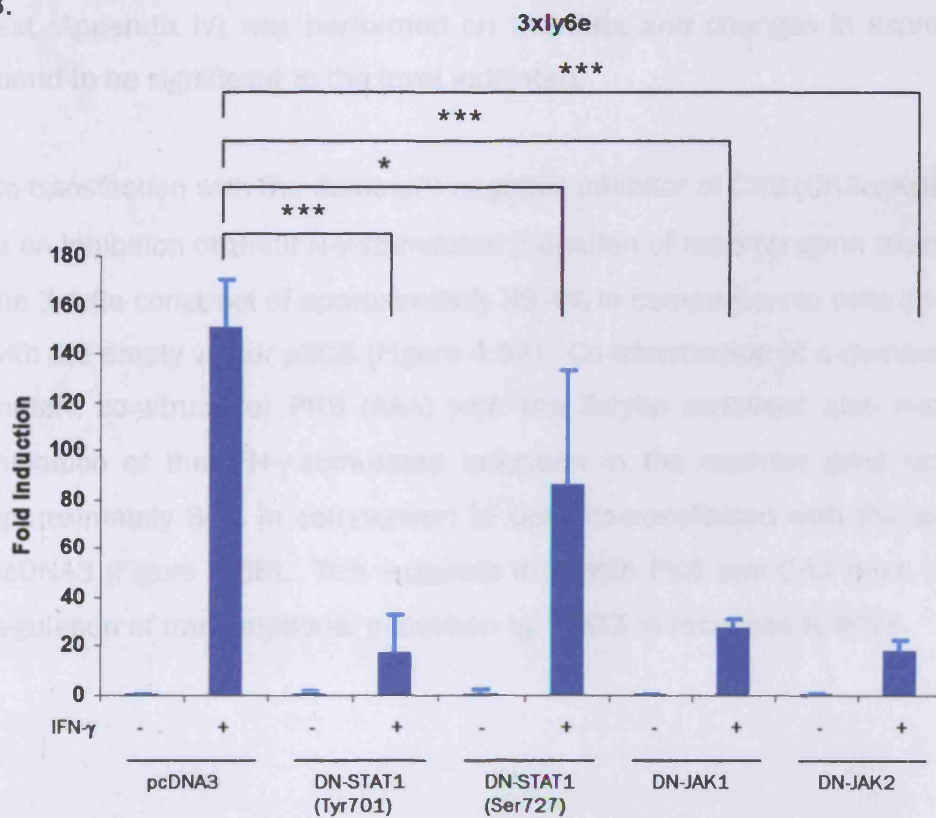
As described previously in Section 3.5.4, Hep3B cells were transfected with the luciferase reporter construct MCP[213]Luc and the response to IFN-γ treatment (6h) analysed. The reporter construct was co-transfected with a plasmid specifying for dominant negative mutants of JAK1 and JAK2 and dominant negative forms of STAT1 mutated at the phosphorylation sites Tyrosine 701 or Serine 727. Due to the unavailability of the empty plasmid vectors used in these constructs (pRK5 and pRC/CMV), pcDNA3 (a similar plasmid containing a CMV promoter region) was co-transfected in control cells. All transfections were carried out with samples in triplicate for at least three independent experiments. Results are presented as the mean fold induction (basal level assigned as 1) (±SD) except where stated otherwise. A standard statistical t-test (Appendix IV) was performed on the data and changes in expression were found to be significant to the level indicated.

Co-transfection of dominant negative JAK1 and JAK2 with MCP[213]Luc resulted in a decrease in the induction by IFN-γ of 75.8% and 92.6% respectively. The repression for the induction of the 3xly6e construct was 82% and 88.2% respectively. The STAT1 (Y701F) dominant negative plasmid inhibited expression from the 3xly6e promoter by approximately 89% but from the MCP[213]Luc promoter by only 46.5% suggesting that other mechanisms may also exist for the regulation of MCP-1 by IFN-γ. The STAT1 (S727A) plasmid reduced the IFN-γ mediated induction of MCP[213]Luc by 36.5% and 3xly6e by 42.8% (Figure 4.4).

A.



B.



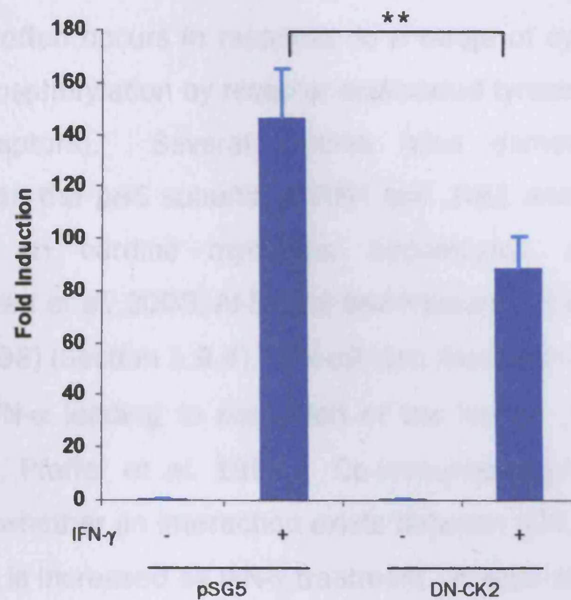
4.3 ROLE OF PI3K AND CK2 IN THE REGULATION OF JAK-STAT SIGNALLING IN RESPONSE TO IFN-γ

4.3.1 Effect of dominant negative inhibitors of CK2 and PKB on the activation of the 3xly6e promoter by IFN-γ

In order to investigate whether CK2 or the downstream effector of PI3K, PKB, has a role in the promoter binding of STAT1 in response to IFN-γ treatment, dominant negative plasmids for each kinase were co-transfected with the GAS-regulated luciferase reporter construct, 3xly6e. Hep3B cells were transfected with 3xly6e as previously described (Section 3.5.4) and the response to IFN-γ treatment (6h) analysed. The reporter construct was co-transfected with plasmids specifying for dominant negative mutants of CK2 and PKB (Section 2.4.5) or with the empty plasmid vectors pSG5 and pcDNA3 respectively. Results are presented as the mean fold induction (basal level assigned as 1) (±SD). A standard statistical T-test (Appendix IV) was performed on the data and changes in expression were found to be significant to the level indicated.

Co-transfection with the dominant negative inhibitor of CK2 (CK2α-K68A) resulted in an inhibition of the IFN-γ-stimulated induction of reporter gene expression from the 3xly6e construct of approximately 39.4% in comparison to cells co-transfected with the empty vector pSG5 (Figure 4.5A). Co-transfection of a dominant negative mutant construct for PKB (AAA) with the 3xly6e construct also resulted in an inhibition of the IFN-γ-stimulated induction in the reporter gene expression of approximately 57% in comparison to cells co-transfected with the empty vector pcDNA3 (Figure 4.5B). This suggests that both PKB and CK2 have a role in the regulation of transcriptional activation by STAT1 in response to IFN-γ.

A.



B.

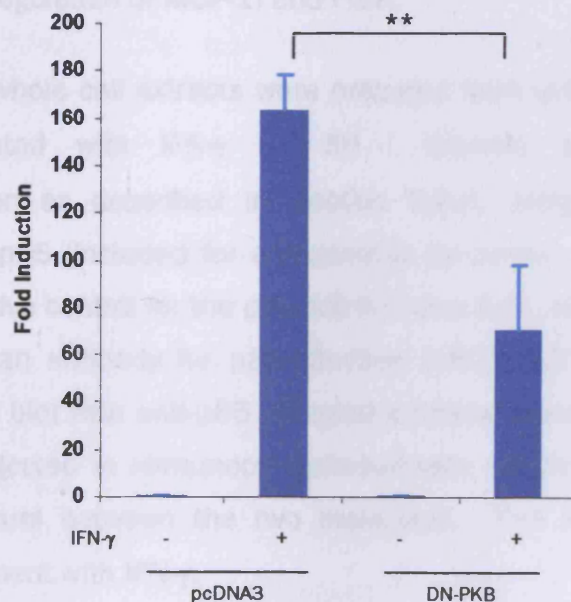


Figure 4.5 Effect of dominant negative inhibitors of PKB and CK2 on the stimulation of 3xly6e promoter activity by IFN-γ

Hep3B cells were transfected with 3xly6e and plasmid constructs specifying for a dominant negative form of CK2α (A) or PKB (B) and the vectors pSG5 and pcDNA3 respectively. Transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration and presented as the mean fold induction (±SD) in response to IFN-γ, in relation to basal expression, from three independent experiments. **P<0.01.

4.3.2 Co-immunoprecipitation of JAK2 and PI3K p85 subunit

Activation of PI3K often occurs in response to a range of cytokines and growth factors through phosphorylation by receptor-associated tyrosine kinases (*e.g.* IL-2 and GM-CSF receptors). Several studies have demonstrated functional interactions between the p85 subunit of PI3K and JAK1 and JAK2, inducible by various cytokines in cardiac myocytes, hepatocytes, macrophages, and neutrophils (Okugawa *et al.* 2003, Al-Shami and Naccache 1999, Oh *et al.* 1998, Yamauchi *et al.* 1998) (Section 1.9.4). IFN-αR also associates with PI3K following stimulation with IFN-α leading to activation of the kinase (Thyrell *et al.* 2004, Uddin *et al.* 2000, Pfeffer *et al.* 1997). Co-immunoprecipitation analysis was used to determine whether an interaction exists between p85 and JAK2 in J774.2 cells and if binding is increased by IFN-γ treatment. If such an interaction existed it would provide a potential mechanistic link between JAK2 (that is essential for IFN-γ-mediated upregulation of MCP-1) and PI3K.

Phosphatase-free whole cell extracts were prepared from untreated J774.2 cells or those stimulated with IFN-γ for 3h. Extracts were subjected to immunoprecipitation as described in Section 2.6.6, using antibodies raised against JAK2 and p85 (included for comparative purposes) or with no antibody (No Ab), as a negative control for the procedure (Table 2.9). Western blot analysis carried out using an antibody for p85 (Section 2.6.7-2.6.8). As illustrated in Figure 4.6 western blot with anti-p85 revealed a protein band of the appropriate size in lysates subjected to immunoprecipitation with anti-JAK2, suggesting that an interaction occurs between the two molecules. This interaction was not increased by treatment with IFN-γ.

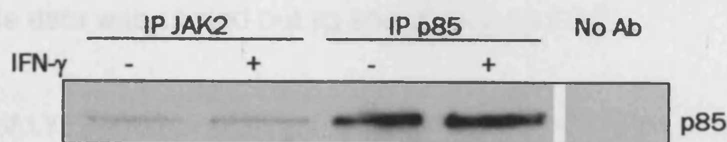


Figure 4.6 Constitutive interaction between JAK2 and p85 subunit of PI3K

J774.2 cells were treated with IFN-γ for 3h and phosphatase-free whole cell protein extracts were prepared. Immunoprecipatation was carried out with anti-JAK2 and anti-p85 antibodies and western blot performed using an antibody specific for p85. No Ab denotes an extract immunoprecipitated with A/G agarose beads only (no antibody). Results are representative of four independent experiments.

4.3.3 Time course for STAT1 phosphorylation by IFN-γ at residues Tyrosine 701 and Serine 727

STAT1 is activated in response to IFN-γ through phosphorylation at Tyrosine 701 by receptor-associated JAKs. This subsequently leads to STAT1 dimerisation and translocation to the nucleus. Maximal activation also requires phosphorylation at Serine 727 although the role and mechanism of phosphorylation at this site is less well understood. The phosphorylation of STAT1 at both tyrosine and serine was analysed in J774.2 cells in response to IFN-γ.

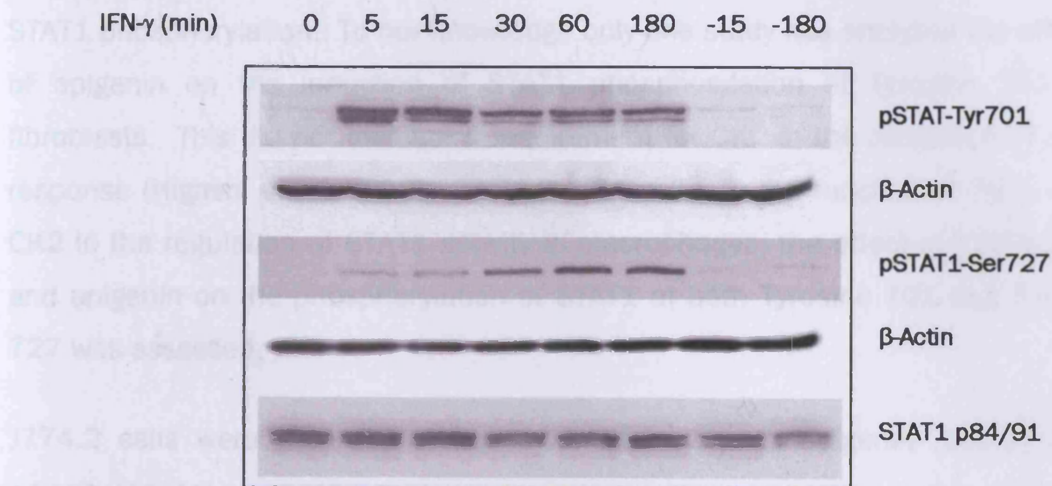
Cells of the J774.2 cell line were stimulated with IFN-γ for periods of 5min, 15min, 30min, 1h and 3h and phosphatase-free whole cell extracts were subsequently prepared from treated cells and untreated controls (at 0h, 15min and 3h). Proteins (10μg for total STAT1, 40μg for phospho-specific antibodies) were separated by SDS-PAGE and transferred to nitrocellulose membrane by immunoblotting (Section 2.6.7). Membranes were probed using antibodies against total STAT1 p84/p91, pSTAT1 (Tyr701) and pSTAT1 (Ser727) (Table 2.9). Following immunodetection, membranes were re-probed with antibodies specific for β-actin to ensure the equal loading of samples.

As shown in Figure 4.7 STAT1 (α and β isoforms, predominantly α) was phosphorylated at Tyrosine 701 after 5min stimulation with IFN-γ and at subsequent time points. Phosphorylation of STAT1 at Serine 727 could also be observed after 5min reaching maximal levels after 30min and at subsequent time points. The amount of total STAT1 protein present did not vary. Densitometric analysis of the data was carried out as shown (Figure 4.7B).

4.3.4 Effect of LY294002 and apigenin on the phosphorylation of STAT1

Previous studies have investigated the effect of the PI3K inhibitor LY294002 on the phosphorylation of STAT1 in a variety of cell lines. LY294002 has been found, in fibroblasts, epithelial and mesangial cells, to inhibit the IFN-γ-induced phosphorylation of STAT1 at Serine 727, so implicating PI3K in the regulation of

A.



B.

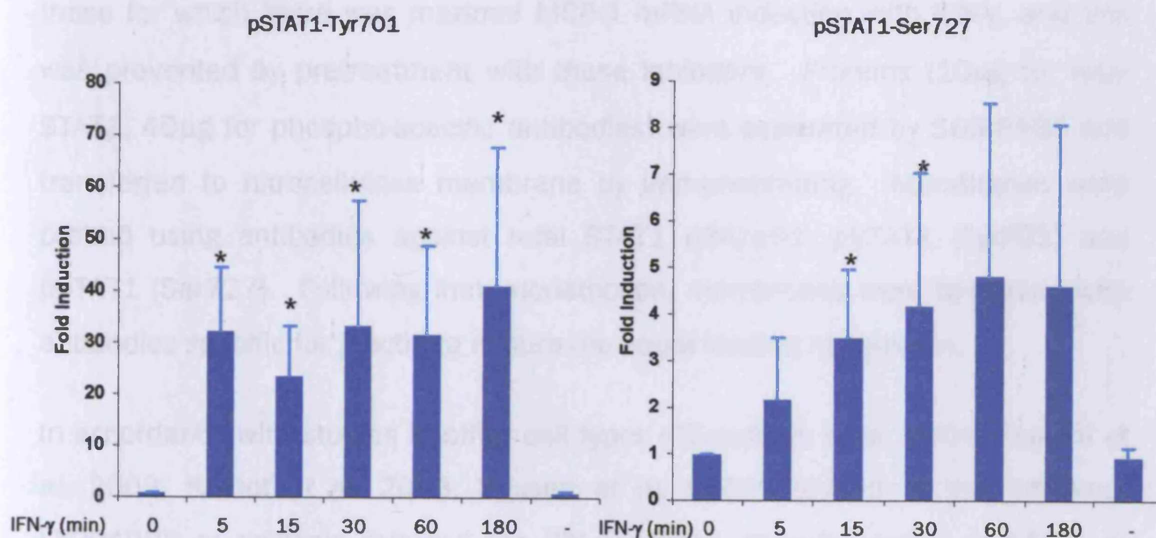


Figure 4.7 Effect of IFN-γ treatment on the phosphorylation of STAT1 at Tyrosine 701 and Serine 727

J774.2 cells were treated with IFN-γ for the times indicated and phosphatase-free whole cell protein extracts were prepared. Western blot analysis was carried out using antibodies specific for: pSTAT1 Tyr701; pSTAT1 Ser727; β-Actin; STAT1 p84/91 (A). Densitometric analysis was carried out (mean ±SD) from at least two independent experiments (B). *P<0.05

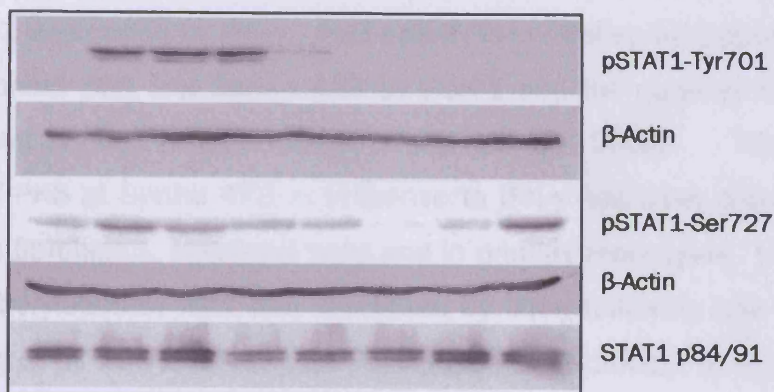
STAT1 at this site (Choudhury *et al.* 2004, Kristof *et al.* 2003, Nguyen *et al.* 2001). Few studies have dealt with the potential role of CK2 in the regulation of STAT1 phosphorylation. To our knowledge only one study has analysed the effect of apigenin on the induction of STAT1 phosphorylation at Tyrosine 701 in fibroblasts. This did not indicate a requirement for CK2 in the mediation of this response (Higashi *et al.* 2003). To further investigate the function of PI3K and CK2 in the regulation of STAT1 activity in macrophages, the effect of LY294002 and apigenin on the phosphorylation of STAT1 at both Tyrosine 701 and Serine 727 was assessed.

J774.2 cells were pre-treated with LY294002 (20μM), apigenin (20μM) and AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which phosphatase-free whole cell extracts were prepared. These concentrations and time point were chosen as those for which there was maximal MCP-1 mRNA induction with IFN-γ, and this was prevented by pretreatment with these inhibitors Proteins (10μg for total STAT1, 40μg for phospho-specific antibodies) were separated by SDS-PAGE and transferred to nitrocellulose membrane by immunoblotting. Membranes were probed using antibodies against total STAT1 p84/p91, pSTAT1 (Tyr701) and pSTAT1 (Ser727). Following immunodetection, membranes were re-probed with antibodies specific for β-actin to ensure the equal loading of samples.

In accordance with studies in other cell types (Choudhury *et al.* 2004, Higashi *et al.* 2003, Kristof *et al.* 2003, Nguyen *et al.* 2001), neither of the inhibitors LY294002 or apigenin reduced the IFN-γ-induced phosphorylation of STAT1 at Tyrosine 701, in contrast to AG490. However the phosphorylation of Serine 727 was inhibited by apigenin and AG490 and, to a slightly lesser extent, LY294002 In two of three experiments, the control extract treated with AG490 alone, displayed an increased phosphorylation of Serine 727, the pathways involved in this are uncertain. The amount of total STAT1 protein did not vary (Figure 4.8A). Densitometric analysis was carried out as shown (Figure 4.8B). These findings correlate with the reduction in the activation of 3xly6e by IFN-γ in co-transfections with dominant negative inhibitors of PKB and CK2 (Figure 4.5) and support a role for these kinases in the activation of STAT1 by the cytokine.

A.

IFN-γ	-	+	+	+	+	-	-	-
LY294002	-	-	+	-	-	+	-	-
Apigenin	-	-	-	+	-	-	+	-
AG490	-	-	-	-	+	-	-	+



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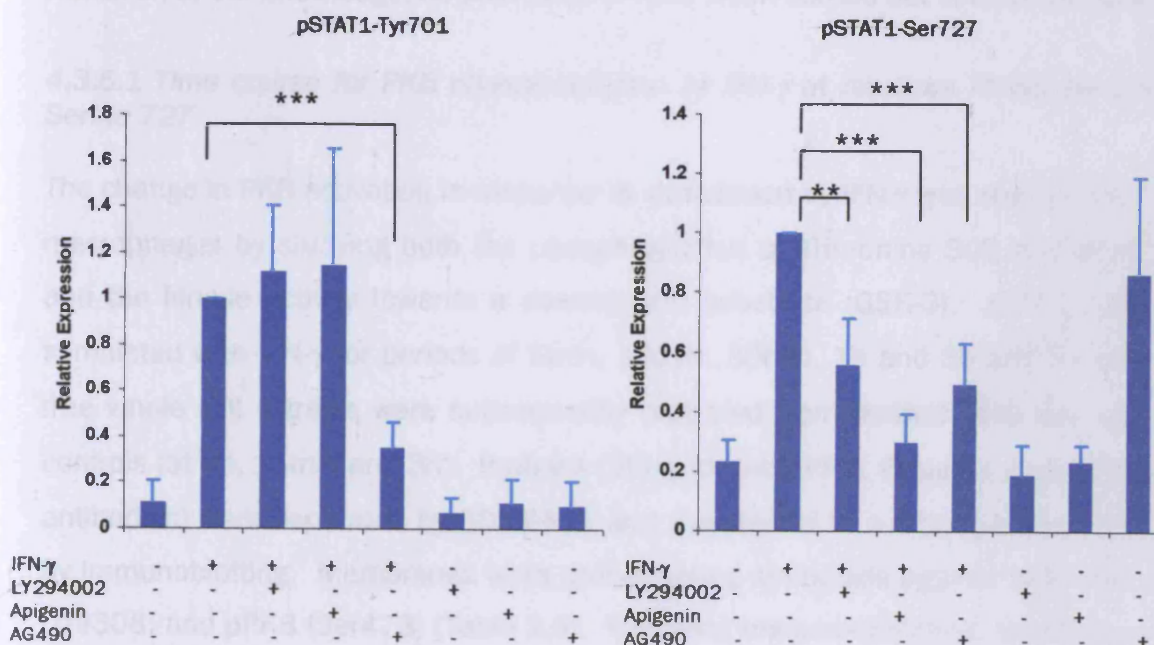


Figure 4.8 Inhibition of IFN-γ-induced phosphorylation of STAT1 at Serine 727 by LY294002 and Apigenin

J774.2 cells were pretreated with LY294002 (20μM), apigenin (20μM), AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Phosphatase-free whole cell protein extracts were prepared and western blot analysis was carried out using antibodies specific for: pSTAT1 Tyr701; pSTAT1 Ser727; β-Actin; STAT1 p84/91 (A). Densitometric analysis was carried out as shown and presented as the relative expression (mean ±SD) from at least three independent experiments (B). **$P<0.01$; ***$P<0.001$.

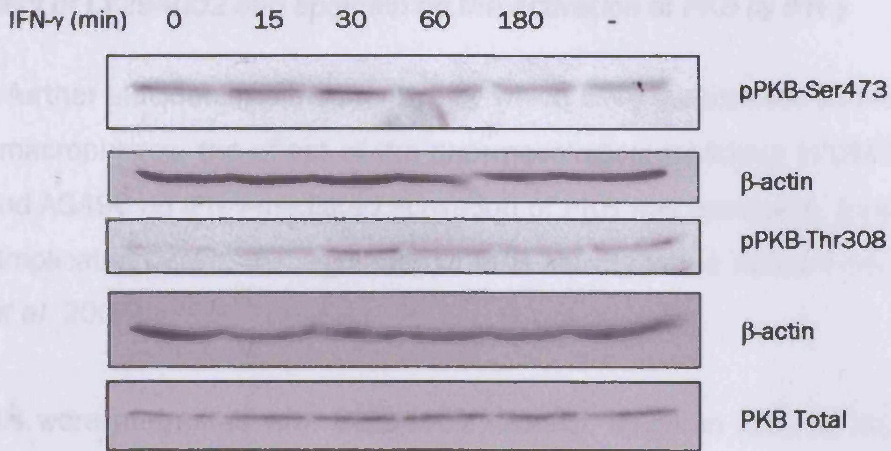
4.3.5 Stimulation of PKB activity by IFN-γ

The use of the pharmacological inhibitor of PKB activity SH6, and a dominant negative construct, demonstrated a role for PKB as a likely downstream effector of PI3K in the regulation of MCP-1 expression by IFN-γ. PKB activity is controlled by phosphorylation of the residues Threonine 308 and Serine 473 by PDK-1 and the putative kinase PDK-2 respectively (Bayascas and Alessi 2005, Yang *et al.* 2004). The enhanced phosphorylation of PKB at Serine 473 in response to IFN-γ has been demonstrated in previous studies in fibroblasts, microglial cells and in primary monocytes. In all of these studies the phosphorylation of PKB was increased by IFN-γ following less than 30min stimulation (Hwang *et al.* 2004, Navarro 2003, Nguyen *et al.* 2001). In human primary monocytes this effect was found to decline at later time points (Navarro *et al.* 2003). However, to our knowledge, no prior studies have been carried out in macrophages.

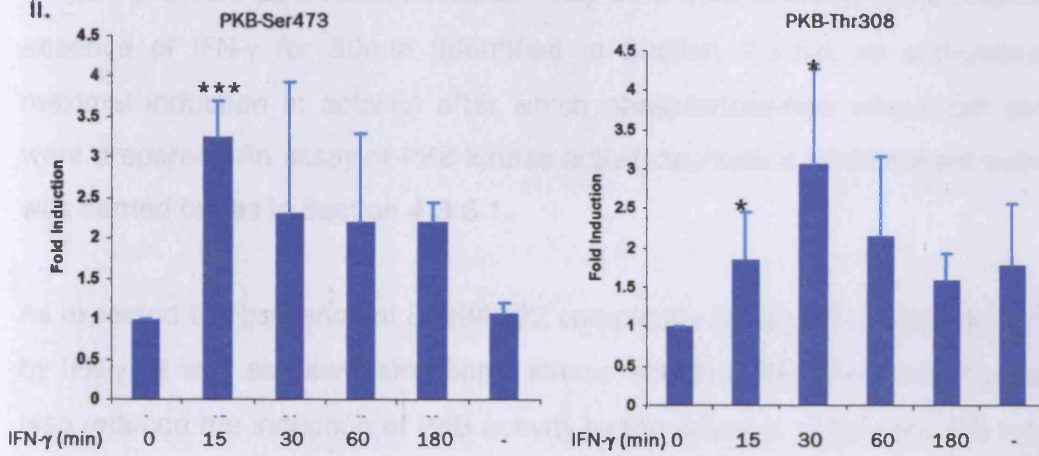
4.3.5.1 Time course for PKB phosphorylation by IFN-γ at residues Threonine 308 and Serine 727

The change in PKB activation in response to stimulation by IFN-γ was analysed in J774.2 macrophages by studying both the phosphorylation at Threonine 308 and Serine 473 and the kinase activity towards a downstream substrate (GSK-3). J774.2 cells were stimulated with IFN-γ for periods of 5min, 15min, 30min, 1h and 3h and phosphatase-free whole cell extracts were subsequently prepared from treated cells and untreated controls (at 0h, 15min and 3h). Proteins (20μg for total PKB, 60μg for phospho-specific antibodies) were separated by SDS-PAGE and transferred to a nitrocellulose membrane by immunoblotting. Membranes were probed using antibodies against total PKB, pPKB (Thr308) and pPKB (Ser473) (Table 2.9). Following immunodetection, membranes were reprobed with an antibody specific for β-actin to ensure the equal loading of samples. As shown in Figure 4.9A PKB was phosphorylated maximally at both Thr308 and Ser473 after approximately 15-30min stimulation with IFN-γ and declined at subsequent time points. The amount of total PKB protein present does not vary. An assay of PKB kinase activity towards a recombinant substrate (Section 2.6.5) was carried out using phosphatase-free whole cell extracts prepared as above. The profile of PKB activation by IFN-γ over time correlated with that of the protein phosphorylation at Thr308 and Ser473 reaching maximal levels at 30min (Figure 4.9B). Densitometric analysis was carried out as shown.

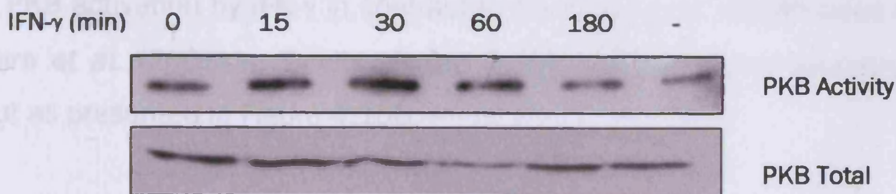
A.i.



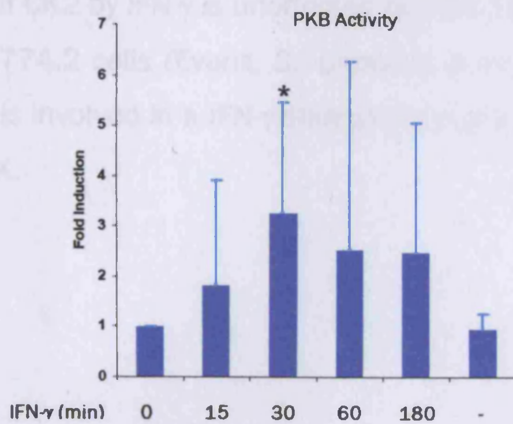
ii.



B.i



ii



4.3.5.2 Effect of LY294002 and apigenin on the activation of PKB by IFN-γ

In order to further elucidate the mechanism by which IFN-γ induces the activation of PKB in macrophages, the effect of the pharmacological inhibitors LY294002, apigenin and AG490 on IFN-γ-mediated activation of PKB was assessed. A recent study has implicated CK2 in the regulation of PKB activity in the Jurkat T-cell line (Di Maira *et al.* 2005).

J774.2 cells were pretreated with LY294002 (20μM), apigenin (20μM), AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 30min (identified in Section 4.3.5.1 as stimulating the maximal induction in activity) after which phosphatase-free whole cell extracts were prepared. An assay of PKB kinase activity towards a recombinant substrate was carried out as in Section 4.3.6.1.

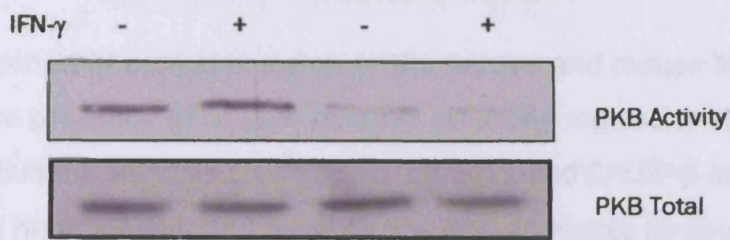
As expected the presence of LY294002 completely blocked the activation of PKB by IFN-γ as well as decreasing basal kinase activity. The JAK2 inhibitor AG490 also reduced the induction of PKB activity by the cytokine supporting the function of JAK2 in IFN-γ-mediated activation of PI3K. The CK2 inhibitor apigenin had no effect on PKB activation by IFN-γ in contrast to the findings of the previous study by Di Maira *et al.* (2005) in T-cells (Figure 4.10). Densitometric analysis was carried out as presented in Figure 4.10B.

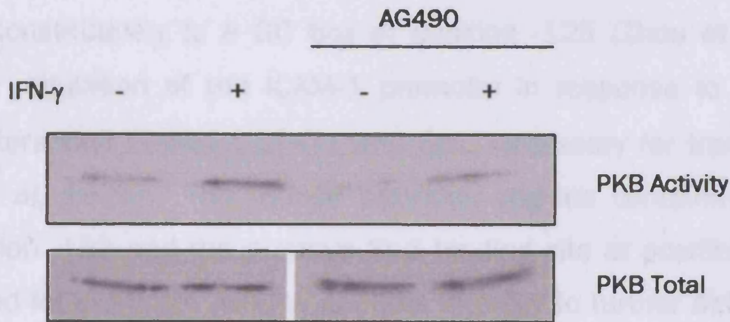
As the activation of CK2 by IFN-γ is unaffected by pretreatment with either AG490 or LY294002 in J774.2 cells (Evans, S., personal communication) these results indicate that CK2 is involved in a IFN-γ-stimulated pathway that is independent of either JAKs or PI3K.

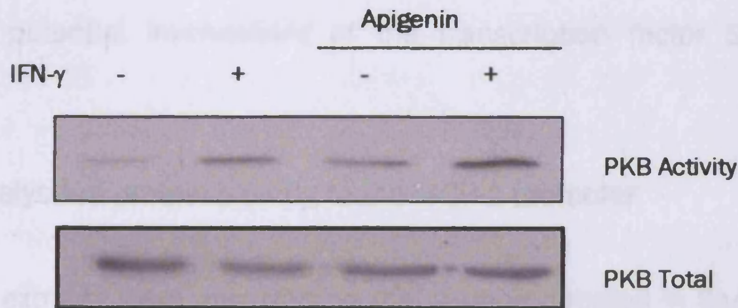
Figure 4.10 Inhibition of IFN-γ-induced activation of PKB by LY294002 and AG490 but not apigenin

J774.2 cells were pretreated with LY294002 (20μM), apigenin (20μM), AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 30min after which phosphatase-free whole cell extracts were prepared. An assay of PKB kinase activity towards a recombinant substrate was performed (A). Densitometric analysis was carried out at shown and presented as relative expression (mean ±SD) from three independent experiments (B). *$P<0.05$; **$P<0.01$.

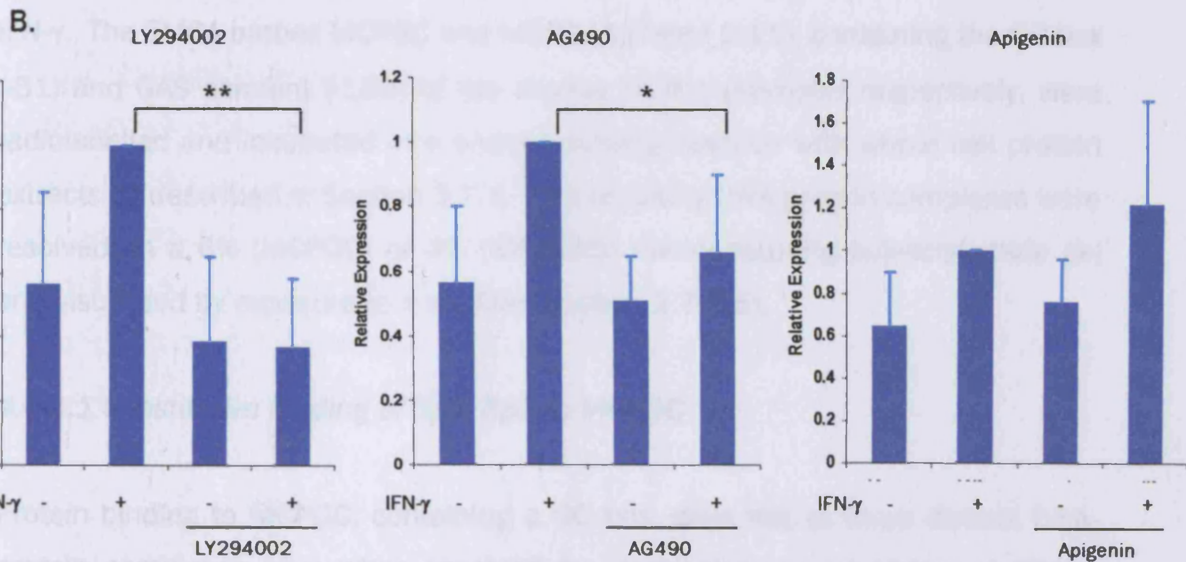
A.







B.



4.4 ANALYSIS OF MCP-1 PROMOTER BINDING

Analyses of the proximal promoter region of the human and mouse MCP-1 gene have revealed the presence of several putative *cis*-acting regulatory elements for transcription factors including Sp1, STAT1, NF-κB, AP-1 and C/EBP-β as illustrated in Figure 3.11. In human astrocytoma cells lines, IFN-γ-inducible binding for STAT1 has been demonstrated to the GAS element at position −213 and Sp1 has been shown to bind constitutively to a GC box at position -125 (Zhou *et al.* 1998). Studies into the regulation of the ICAM-1 promoter in response to IFN-γ have suggested an interaction between STAT1 and Sp1, necessary for transcriptional activity (Look *et al.* 1995). The mouse promoter regions containing the GAS element at position -159 and the putative Sp1 binding site at position -51 were therefore selected for promoter binding analysis in order to further determine the role of STAT1 in the IFN-γ-mediated upregulation of MCP-1 gene expression and to investigate the potential involvement of the transcription factor Sp1 in this response.

4.4.1 EMSA analysis of protein binding to the MCP-1 promoter

Nuclear protein extracts were prepared by the method detailed in Section 2.6.3, from untreated J774.2 cells and those stimulated for 30min or 3h with murine IFN-γ. The EMSA probes MCPGC and MCPGAS (Table 2.11), containing the GC box (-51) and GAS element (-159) of the murine MCP-1 promoter respectively, were radiolabelled and incubated in a protein binding reaction with whole cell protein extracts as described in Section 2.7.1. The resulting DNA-protein complexes were resolved on a 6% (MCPGC) or 4% (MCPGAS) non-denaturing polyacrylamide gel and visualised by exposure to X-ray film (Section 2.7.1.5).

4.4.1.1 Constitutive binding of Sp1/Sp3 to MCPGC

Protein binding to MCPGC, containing a GC box, gave rise to three distinct DNA-protein complexes (a-c) when resolved by electrophoresis as shown in Figure 4.11. This binding was competed by an excess of the unlabelled probe and by the

consensus sequence for Sp1 binding but not by a consensus sequence for AP-1 (Section 2.7.1.4). Incubation with antibodies for Sp1 inhibited the formation of complex a and incubation with anti-Sp3 prevented the formation of complexes b and c. In addition, a DNA-protein-antibody supershift complex was obtained with these two antibodies which is particulary prominent with the Sp3 antibody (Section 2.7.1.4) (Figure 4.11A). The binding of Sp1 and Sp3 to MCP-GC was unaffected by IFN-γ (Figure 4.11B). Densitometric analysis of this data is also presented.

4.4.1.2 IFN-γ-induced protein binding to MCPGAS

Figure 4.12 illustrates that protein binding to the GAS element of the proximal MCP-1 promoter is inducible by IFN-γ after stimulation for 30min and declines at 3h. Two DNA-protein complexes were present (a and b) the binding of which was competed by an excess of the unlabelled probe and a consensus STAT1-binding site but not by a double stranded oligonucleotide for the NF-κB consensus element, indicating that the binding is sequence specific. Although further studies are required to confirm the identity of each band, band a is at a position consistent with that seen for STAT1 binding in previous studies (Zhou *et al.* 1998). A supershift antibody was unavailable in the laboratory for STAT1 at this time. Densitometric analysis of this data was carried out as shown (Figure 4.12B).

These findings confirm those of Zhou *et al.* (1998) who determined, in astrocytoma cells, that Sp1 bound constitutively to the MCP-1 promoter and the STAT1 binding was maximally induced at 30min and decreased by 5h. They also showed through mutation of the GAS and GC elements that the GAS element was necessary for the upregulation of MCP-1 expression from a reporter plasmid by IFN-γ and that inactivation of the GC box reduced the basal expression from the promoter without affecting IFN-γ-inducibility (Zhou *et al.* 1998).

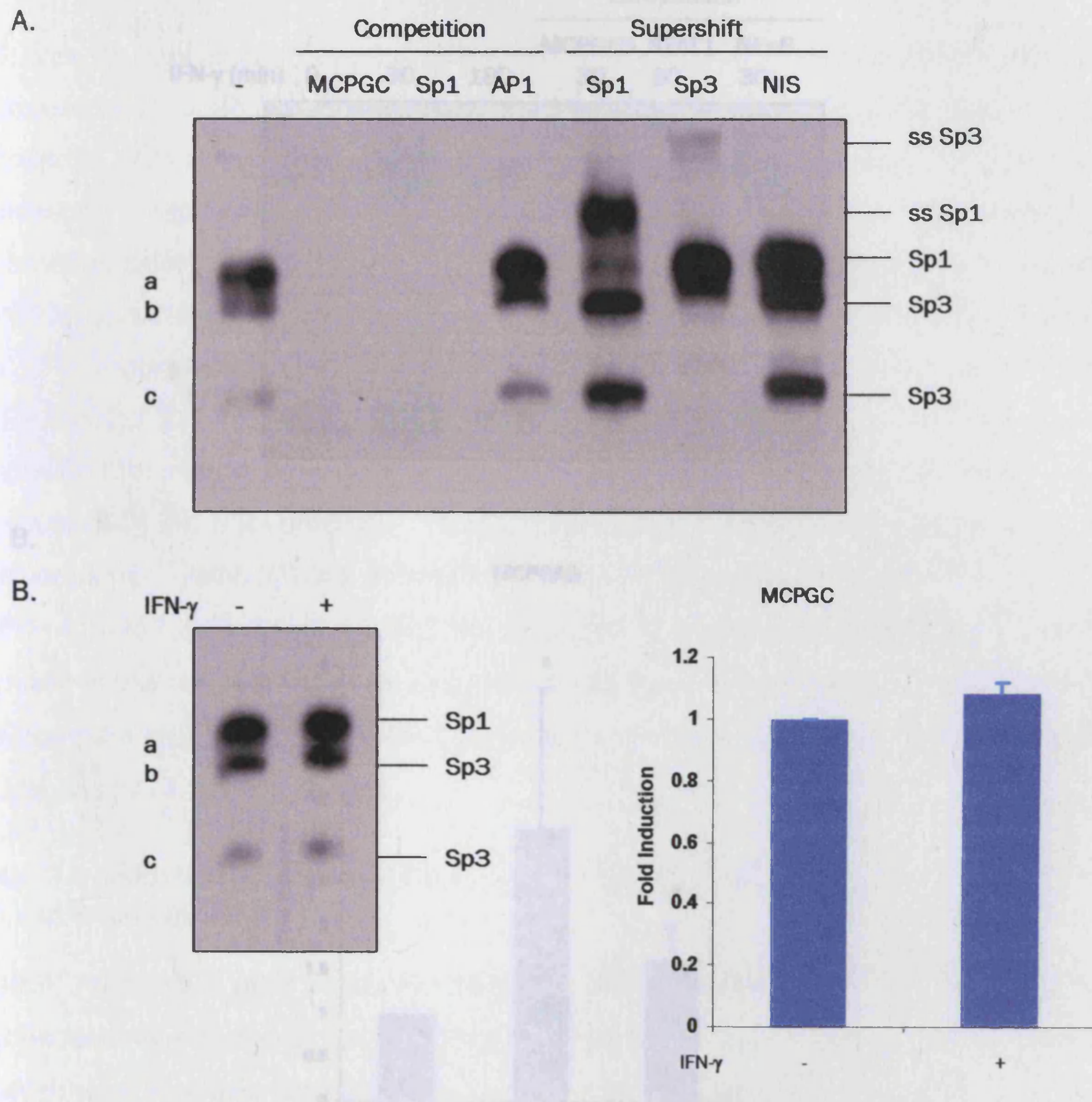
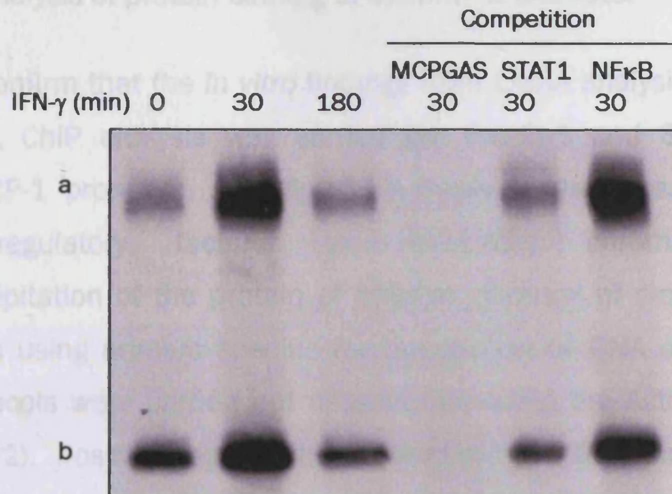


Figure 4.11 Constitutive binding of Sp1/Sp3 to MCPGC

J774.2 cells were cultured in the presence or absence of IFNγ for 3h and nuclear extracts prepared. EMSA analysis was carried out for extracts using the radiolabelled probe MCPGC. Competition studies were carried out using 250x molar excess of unlabelled probe and unlabelled Sp1 and AP-1 consensus sequences. Supershift was performed using anti-Sp1 and anti-Sp3 antibodies. Results are representative of two independent experiments (A). Densitometric analysis shows the mean fold induction with IFN-γ (±SD) from three experiments (B).

A.



B.

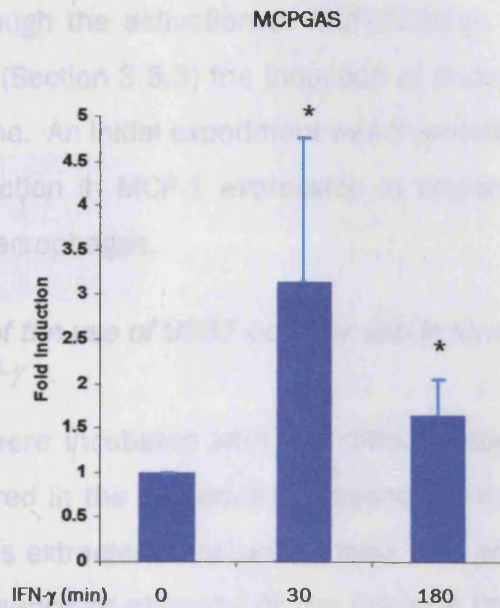


Figure 4.12 IFN-γ induces transient binding to MCPGAS

J774.2 cells were treated with IFNγ for times of 0h, 30min and 3h and nuclear extracts prepared. EMSA analysis was carried out for extracts using the radiolabelled probe MCPGAS. Competition studies were carried out using 250x molar excess of unlabelled probe and unlabelled GAS and NF-κB consensus sequences (A). Densitometric analysis is presented as the mean fold induction with IFN-γ (±SD) from three experiments (B). *$P<0.05$.

4.4.2 ChIP analysis of protein binding to the MCP-1 promoter

In order to confirm that the *in vitro* findings from EMSA analysis occur in the cellular environment, ChIP analysis was carried out for Sp1 and STAT1 binding to the proximal MCP-1 promoter. Briefly, ChIP involves: the cross-linkage of proteins, including regulatory factors, to chromatin; chromatin fragmentation; immunoprecipitation of the protein of interest; reversal of crosslinking; followed by PCR analysis using primers specific for the section of DNA of interest (see Figure 4.13). Protocols were carried out as specified using the Active Motif ChIP-IT™ Kit (Section 2.7.2). Positive and negative control primers supplied by Active Motif were specified for use in human cells and U937 monocytes have previously been used successfully in this protocol. This system was therefore employed in these experiments. Although the activation of MCP[213]Luc has been demonstrated by IFN-γ in U937 cells (Section 3.5.3) the induction of endogenous mRNA has not been shown in this cell line. An initial experiment was therefore carried out to demonstrate a comparable induction in MCP-1 expression in response to IFN-γ between these cells and J774.2 macrophages.

4.4.2.1 Validation of the use of U937 cells for use in studies of the induction of MCP-1 expression by IFN-γ

U937 monocytes were incubated with the differentiating agent PMA for 24h and subsequently cultured in the presence or absence of human IFN-γ for 3h, following which total RNA was extracted. For comparison, RNA was also prepared using cells cultured in the presence or absence of the cytokine that had not be preincubated with PMA. RT-PCR analysis was carried out as before using primers for human MCP-1 and β-actin. PCR products were resolved by agarose gel electrophoresis as shown in Figure 4.14. The expression of MCP-1 was induced by IFN-γ in both monocytes and cells differentiated in the presence of PMA. Notably, the overall level of MCP-1 expression was dramatically increased by PMA treatment. This is consistent with other studies that have found that MCP-1 secretion in increased following the differentiation of human monocytes to macrophages (Fantuzzi *et al.* 1999) although a study by Gruss *et al.* (1994). has shown the opposite effect. These results indicate that U937 cells are suitable for use in the analysis of MCP-1 promoter binding in response to IFN-γ.

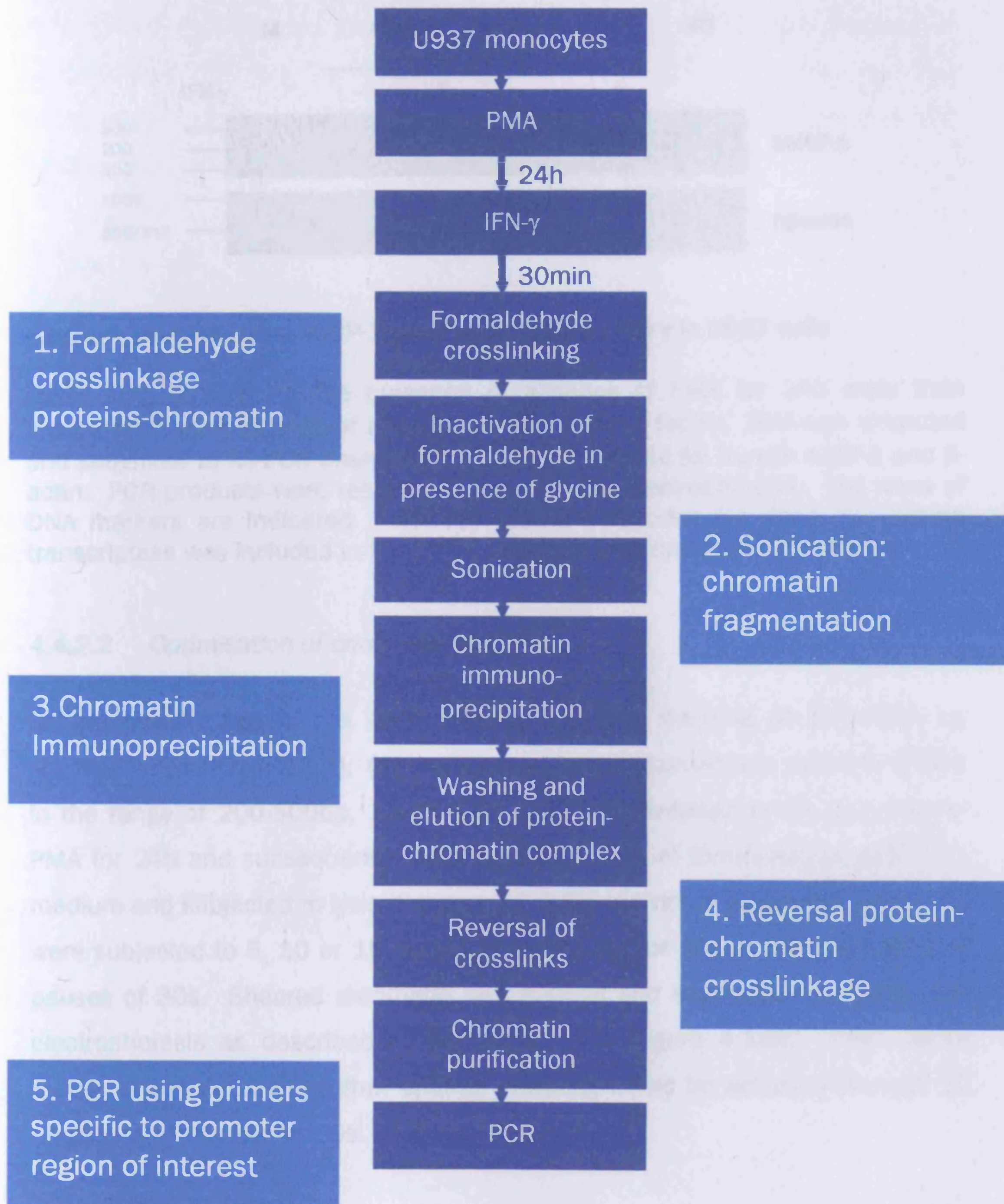


Figure 4.13 Stages of ChIP protocol

U937 monocytes were differentiated in the presence of PMA for 24h and subsequently treated with IFN-γ for 30min. Cells were then subjected to ChIP analysis as follows:

1. Formaldehyde crosslinkage of proteins to chromatin
2. Sonication: chromatin fragmentation
3. Immunoprecipitation of the protein of interest
4. Reversal of protein-chromatin crosslinkage
5. PCR analysis using primers specific for promoter region of interest

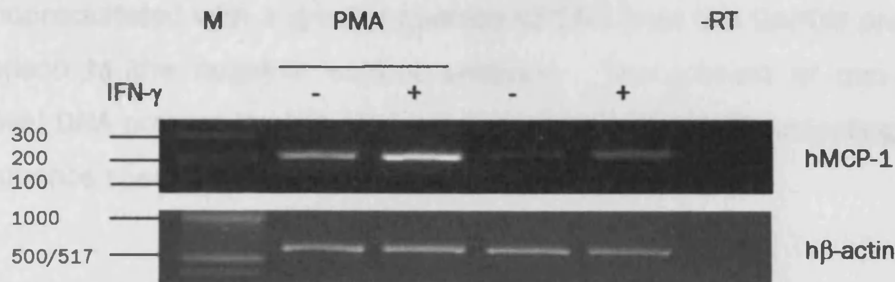


Figure 4.14 Induction of MCP-1 gene expression by IFN-γ in U937 cells

U937 cells cultured in the presence or absence of PMA for 24h were then incubated in the presence or absence of human IFN-γ for 3h. RNA was extracted and subjected to RT-PCR analysis using primers specific for human MCP-1 and β-actin. PCR products were resolved by agarose gel electrophoresis. The sizes of DNA markers are indicated. -RT represents a reaction for which no reverse transcriptase was included in the cDNA synthesis step (using untreated RNA).

4.4.2.2 *Optimisation of chromatin shearing*

An important stage in the ChIP procedure is the shearing of chromatin by sonication (Section 2.7.2.3). Chromatin must be fragmented into sections of DNA in the range of 200-500bp. U937 cells were differentiated in the presence of PMA for 24h and subsequently fixed by the addition of formaldehyde to culture medium and subjected to lysis (Section 2.7.2.2). Individual aliquots of chromatin were subjected to 5, 10 or 15 pulses of sonication for 20s or 30s separated by pauses of 30s. Sheared chromatin was purified and separated by agarose gel electrophoresis as described in Section 2.7.2.3 (Figure 4.15A). From these results it was determined that optimal shearing would be achieved through 12 periods of sonication for 30s.

To assess the effectiveness of this protocol, ChIP was carried out using the TFIIB antibody supplied by Active Motif for immunopreciptation, alongside a negative control antibody (also supplied). Immunoprecipitates were subjected to PCR analysis using primers for the GAPDH promoter and a region of gDNA between the GAPDH gene and the chromosome condensation-related SMC-associated protein (CNAP1) gene, which should not contain binding sites for any transcription factors, as a negative control PCR (Table 2.5). Products were resolved by agarose gel electrophoresis (Figure 4.15B). As expected the TFIIB antibody was

immunoprecipitated with a greater quantity of DNA from the GAPDH promoter in comparison to the negative control antibody. The amount of non-promoter (negative) DNA present in each immunoprecipitate was equal, indicating that this is a sequence specific effect.

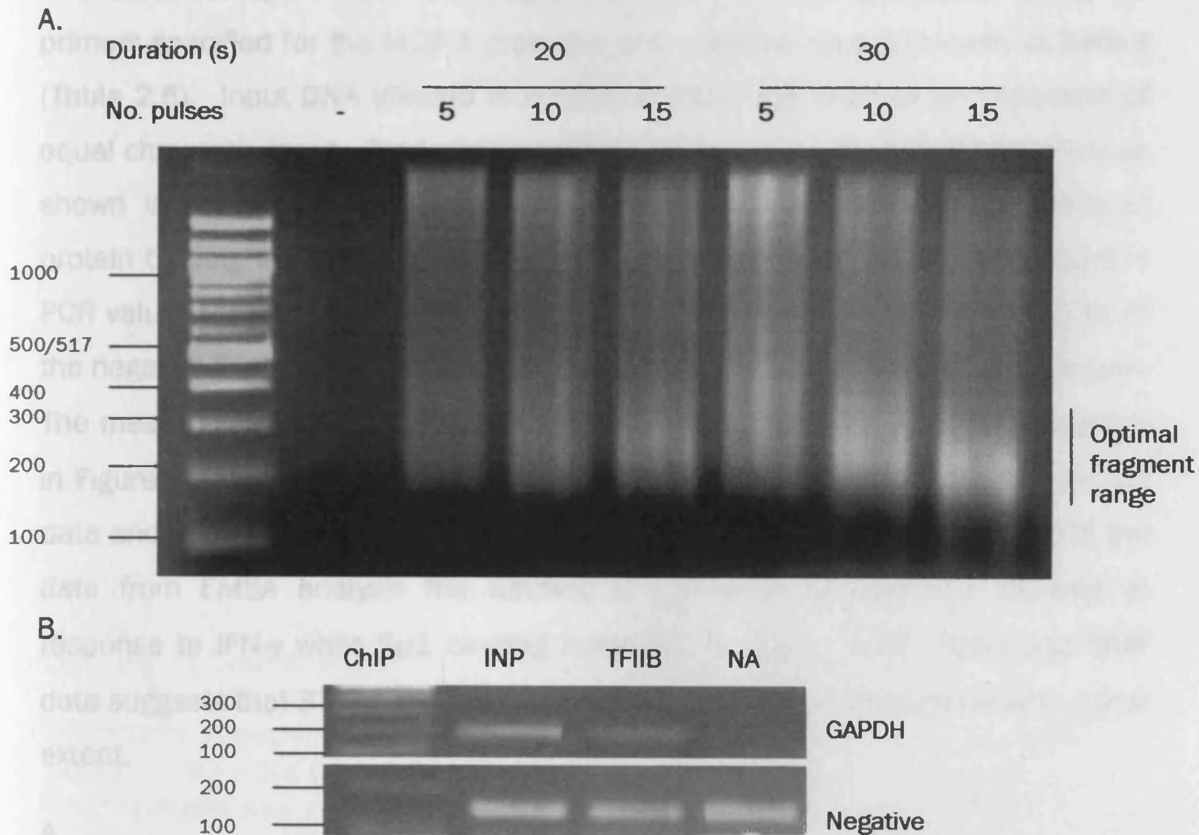


Figure 4.15 Optimisation of ChIP protocol

Aliquots of chromatin were sheared by sonication for the periods indicated, purified and resolved by agarose gel electrophoresis (A). ChIP analysis was performed on chromatin subjected to 12 pulses of sonication of 30s. Immunoprecipitation was carried out using an antibody specific for TFIIB and a negative control IgG (NA) and PCR using primers for a region of the GAPDH promoter and a non-promoter section of gDNA (negative). PCR was also carried out using Input DNA (INP). Products were resolved by agarose gel electrophoresis (B).

4.4.2.3 ChIP analysis of the effect of IFN-γ on Sp1 and STAT1 binding to the MCP-1 promoter

In order to perform ChIP analysis for the binding of STAT1 and Sp1 to the human MCP-1 promoter, oligonucleotide primers (GC-GAS) were generated to amplify the region of the promoter between -289 and -95bp upstream of the transcriptional

start site giving a 194bp product as illustrated in Figure 4.16. U937 cells, differentiated in presence of PMA for 24h, were treated with human IFN-γ for 30min (for which optimal GAS binding was observed in EMSA). ChIP analysis was carried out as in Section 4.4.2.2. Immunoprecipitation was performed using antibodies for Sp1 and STAT1 (Table 2.9) and PCR was carried out using the primers specified for the MCP-1 promoter and negative control primers as before (Table 2.5). Input DNA (diluted in a ratio of 1:10) was used as an indication of equal chromatin input. Products were resolved by agarose gel electrophoresis as shown in Figure 4.17A. Densitometry was carried out and relative levels of protein binding were assessed as follows: normalisation of GC-GAS and negative PCR values for each sample to the input value; normalisation of GC-GAS values to the negative PCR; and subtraction of the negative antibody value for each sample. The mean (±SD) as calculated over three independent experiments is presented in Figure 4.17B. A standard statistical t-test (Appendix IV) was performed on the data and the results were significant to the degree indicated. Consistent with the data from EMSA analysis the binding of STAT1 to GC-GAS was induced in response to IFN-γ while Sp1 binding remained constant. Both EMSA and ChIP data suggests that STAT1 also binds constitutively to the MCP-1 promoter to some extent.

A.

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Tgtttacacaatcctacagttctgctaggcttctatgatgctactattctgcatttgaatgagcaaatggatttaatgca
ttgtcagggagccggccaaagcttgagagctccttcctggctgggaggccccttggaatgtggcctgaaggtaagctggc
agcgagcctgacatgctttcatctagtttcctcgcttccttcctttctgcagttttcgcttcacagaaagcagaatcct
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cctcctcctgcttgactccgccc[GC]tctctccctctgcccgctttcaataag[-95][Rev]aggcagagacagcagc
cagaggaaccgagaggctgagactaacccagaaacatccaattctcaaactgaagctcgcactctcgcctccagcatg[-1]
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B.

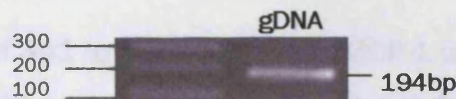


Figure 4.16 Region of the human MCP-1 promoter amplified by PCR using the primers GC-GAS

The position of forward (For) and reverse (Rev) primers and the GC and GAS elements of interest are highlighted (A). gDNA was extracted from untreated U937 cells and subjected to PCR using the primers GC-GAS. The product was resolved by agarose gel electrophoresis (B).

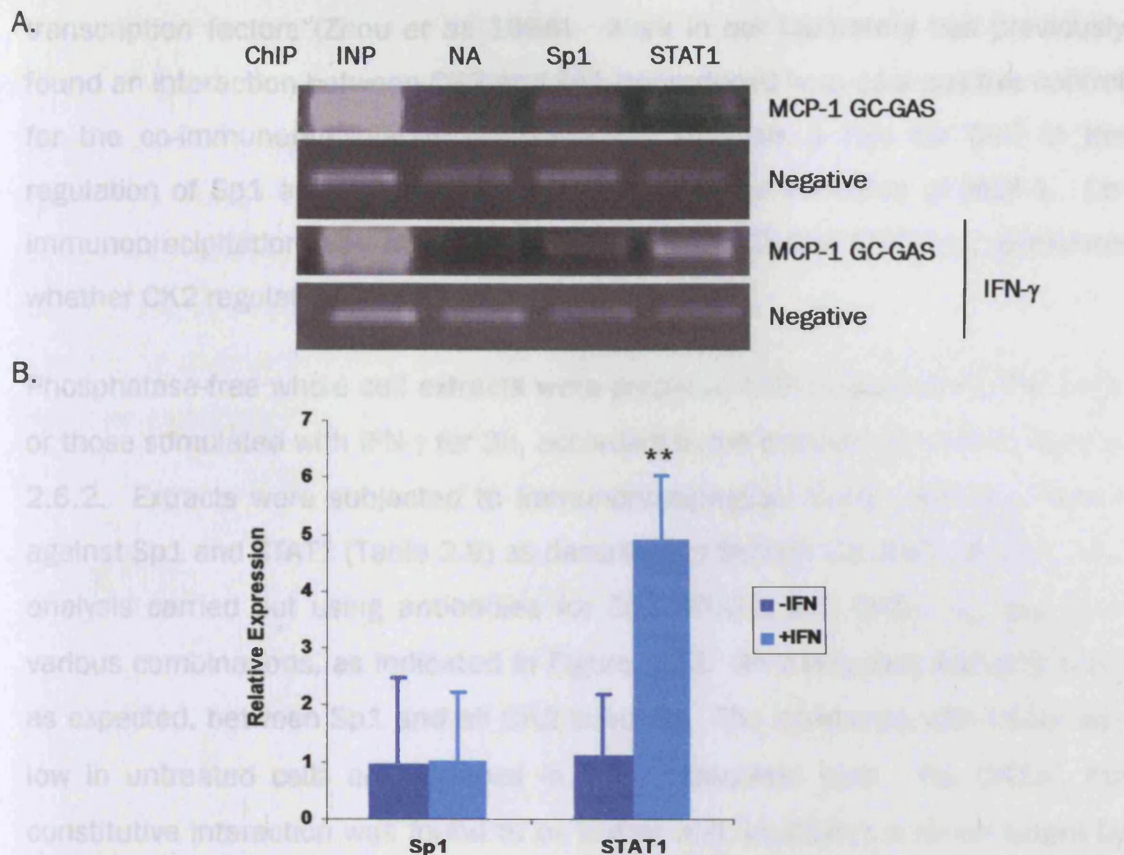


Figure 4.17 ChIP analysis of Sp1 and STAT1 binding to the MCP-1 promoter

U937 cells differentiated in the presence of PMA for 24h were treated with IFN-γ for 30min. Cells were fixed in the presence of formaldehyde and chromatin extracted. ChIP analysis was carried out using antibodies specific for Sp1 and STAT1 and a negative control IgG (NA). PCR primers were specific for a section of the human MCP-1 promoter (MCP-1 GC-GAS) and a region of non-promoter gDNA (negative). PCR was also carried out using Input DNA (INP). Products were resolved by agarose gel electrophoresis (A). Densitometric analysis was carried out and the relative levels of protein binding for Sp1 and STAT1 in comparison to NA (value assigned as 0) are presented in Panel B as mean (±SD) from three experiments. **$P<0.01$.

4.4.3 Co-immunoprecipitation studies analysing the interactions between Sp1, STAT1 and CK2 in response to stimulation with IFN-γ

Constitutive binding of Sp1 to a GC box of the MCP-1 promoter was demonstrated by both EMSA and ChIP analysis in Sections 4.4.1-4.4.2. Co-immunoprecipitation studies were subsequently performed in order to establish whether an interaction exists between Sp1 and STAT1 that may play a part in the IFN-γ response. Such an interaction has previously been demonstrated in relation to the regulation of the expression of the ICAM-1 gene by IFN-γ in human epithelial cells (Look *et al.* 1995) although other studies have failed to show binding between the two

transcription factors (Zhou *et al.* 1998). Work in our laboratory has previously found an interaction between CK2 and Sp1 (reproduced here as a positive control for the co-immunoprecipiation protocol) that suggests a role for CK2 in the regulation of Sp1 activity that may be relevant to the induction of MCP-1. Co-immunoprecipitation was also analysed between CK2 and STAT1 to determine whether CK2 regulates STAT1 in a similar mechanism.

Phosphatase-free whole cell extracts were prepared from untreated J774.2 cells, or those stimulated with IFN-γ for 3h, according to the protocol detailed in Section 2.6.2. Extracts were subjected to immunoprecipitation using antibodies raised against Sp1 and STAT1 (Table 2.9) as described in Section 2.6.5 and western blot analysis carried out using antibodies for Sp1, STAT1, and CK2α, α', and β, in various combinations, as indicated in Figure 4.18. An interaction was observed, as expected, between Sp1 and all CK2 subunits. The interaction with CK2α was low in untreated cells and induced in IFN-γ stimulated cells. For CK2α', the constitutive interaction was found to be higher and induced to a lesser extent by IFN-γ. CK2β appears to be constitutively bound to Sp1. In contrast, no interaction was seen between CK2 and STAT1. An IFN-γ inducible interaction was demonstrated between Sp1 and STAT1 as previously shown by Look *et al.* (1995) in epithelial cells. Densitometric analysis was carried out as shown for the fold induction of Sp1-STAT1 by IFN-γ (Figure 4.18B).

4.4.4 Role of other transcription factors in the regulation of MCP-1 expression by IFN-γ

The MCP-1 promoter contains several putative *cis*-acting regulatory elements for transcription factors including Sp1, STAT1, NF-κB, AP-1 and C/EBP-β as illustrated in Figure 3.11. Previous studies have indicated a role for all of these factors in the regulation of MCP-1 expression in response to cytokine stimulation (Table 1.7). The putative AP-1 element overlaps the Sp1 site in the proximal promoter and as indicated by EMSA analysis protein binding to this region is not competed by unlabelled double-stranded DNA encoding a consensus AP-1 site (Figure 4.11). A NF-κB binding site is also present in the MCP-GAS EMSA probe but protein binding was not inhibited by an unlabelled NF-κB consensus sequence (Figure

4.12). However another putative κB binding site has been identified in the human MCP-1 promoter that may be responsive to IFN-γ-stimulation. Transcriptional activation of both C/EBP-β and NF-κB has been linked to IFN-γ signalling and may be regulated by both PI3K and CK2 (Section 1.8.2.1). The endogenous inhibitors of C/EBP-β and NF-κB activity, LIP and IκB respectively (Section 2.4.5), were overexpressed in co-transfection studies with MCP[213]Luc in order to assess the role of these transcription factors in the regulation of promoter activation by IFN-γ.

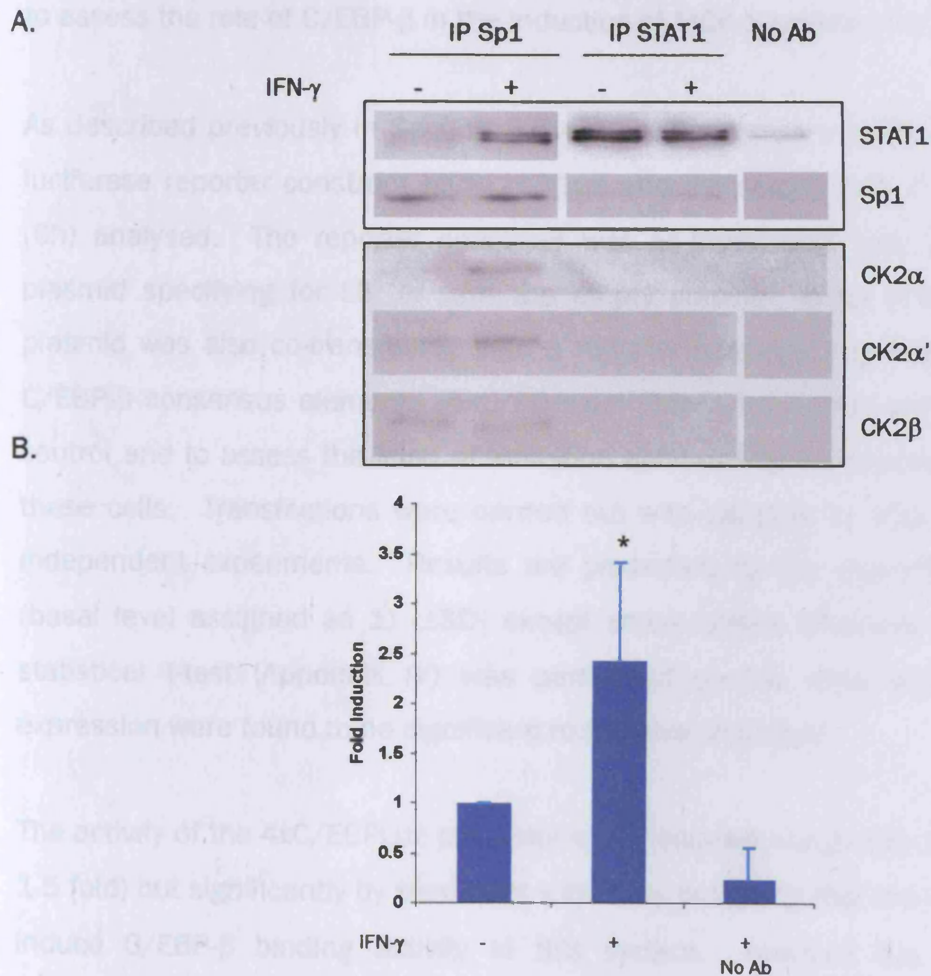


Figure 4.18 Co-immunoprecipitation studies to analyse interactions between Sp1, CK2 and STAT1

Phosphatase-free whole cell protein extracts were immunoprecipatated with anti-Sp1 and anti-STAT1 antibodies and western blot performed using antibodies specific for: CK2 (α, α' and β); Sp1; STAT1. No Ab denotes an extract immunoprecipitated using A/G Agarose beads only (no antibody) (A). Densitometric analysis was carried out and the results are presented as the mean fold induction with IFN-γ (±SD) from four independent experiments (B). *P<0.05.

4.4.4.1 Role of C/EBP-β in the induction of MCP-1 expression by IFN-γ

The C/EBP-β transcript is translated into two major isoforms: liver-enriched activating protein (LAP) (C/EBP-β) and liver-enriched inhibitory protein (LIP). LIP lacks a section of the *trans*-activating domain but retains the ability to dimerise with other isoforms and to bind DNA. Hence LIP is able to act as an endogenous dominant negative repressor of C/EBP-β mediated gene expression (Ramji and Foka 2002). An expression plasmid encoding the *Xenopus* form of LIP was used to assess the role of C/EBP-β in the induction of MCP-1 expression by IFN-γ.

As described previously in Section 3.5.4, Hep3B cells were transfected with the luciferase reporter construct MCP[213]Luc and the response to IFN-γ treatment (6h) analysed. The reporter construct was co-transfected with an expression plasmid specifying for LIP or with the empty plasmid vector pCS2+. The LIP plasmid was also co-transfected with a reporter construct regulated by multiple C/EBP-β consensus elements (4xC/EBPLuc) (Section 2.4.5) to act as a positive control and to assess the level of inhibition achieved by overexpression of LIP in these cells. Transfections were carried out with samples in triplicate for three independent experiments. Results are presented as the mean fold induction (basal level assigned as 1) (±SD) except where stated otherwise. A standard statistical t-test (Appendix IV) was performed on the data and changes in expression were found to be significant to the level indicated.

The activity of the 4xC/EBPLuc promoter was increased marginally (approximately 1.5 fold) but significantly by treatment with IFN-γ indicating that the cytokine does induce C/EBP-β binding activity in this system. However the induction of MCP[213]Luc by IFN-γ was not affected by co-transfection with the LIP expression plasmid. The positive action of LIP was confirmed by its ability to inhibit basal transcription from the 4xC/EBPLuc construct (suppressed by 57%) (Figure 4.19). This suggests that although C/EBP-β binding activity can be increased in response to IFN-γ this mechanism is not likely to be involved in the regulation of MCP-1 expression by the cytokine.

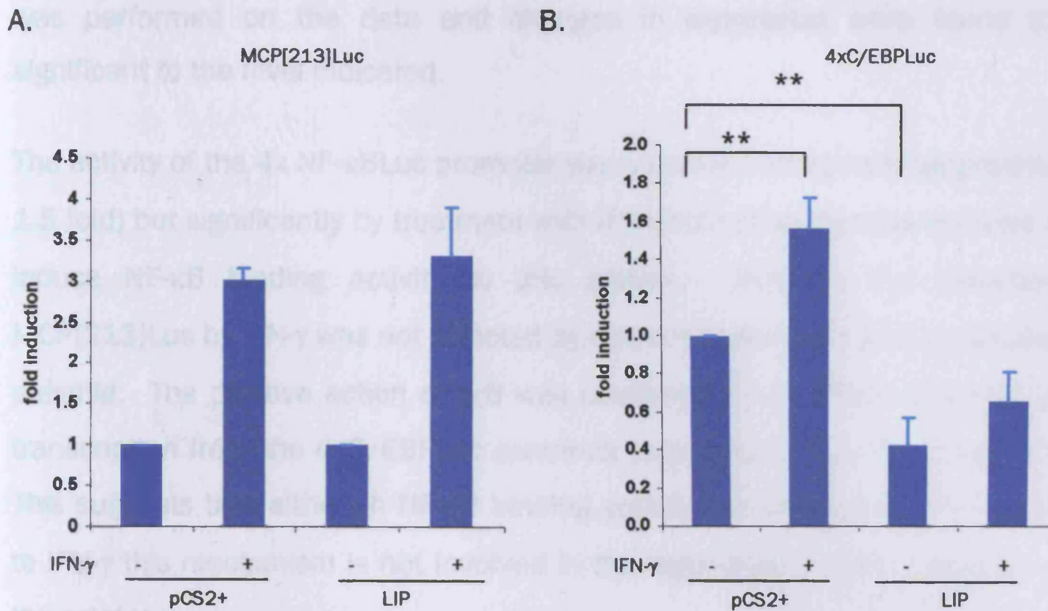


Figure 4.19 Role of C/EBP-β in the induction of MCP-1 expression by IFN-γ

Hep3B cells were transfected with MCP[213]Luc (A) or 4xC/EBPLuc (B) and a plasmid construct expressing LIP or the empty vector pCS2+. Transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration and presented as the mean fold induction (±SD) in response to IFN-γ, in relation to basal expression (assigned as 1), from three independent experiments. In the case of co-transfections for LIP and the 4xC/EBPLuc reporter, counts were normalised to the basal expression of 4xC/EBPLuc transfected with pCS2+. $**P<0.01$

4.4.4.2 Role of NF-κB in the induction of MCP-1 expression by IFN-γ

The function of IκB as an inhibitor of NF-κB activation is discussed in Section 1.8.2.1. As described previously in Section 3.5.4, Hep3B cells were transfected with the luciferase reporter construct MCP[213]Luc and the response to IFN-γ treatment (6h) analysed. The reporter construct was co-transfected with an expression plasmid specifying for IκB or with the empty plasmid vector pcDNA3. The IκB plasmid was also co-transfected with a reporter construct regulated by multiple NF-κB binding elements (4x NF-κBLuc) (Section 2.4.5) to assess the level of inhibition achieved by overexpression of IκB and act as a positive control. Results are presented as the mean fold induction (basal level assigned as 1) (±SD) except where stated otherwise. A standard statistical t-test (Appendix IV)

was performed on the data and changes in expression were found to be significant to the level indicated.

The activity of the 4x NF-κBLuc promoter was increased marginally (approximately 1.5 fold) but significantly by treatment with IFN-γ indicating that the cytokine does induce NF-κB binding activity in this system. However the induction of MCP[213]Luc by IFN-γ was not affected by co-transfection with the IκB expression plasmid. The positive action of IκB was confirmed by its ability to inhibit basal transcription from the 4xC/EBPLuc construct (suppressed by 57%) (Figure 4.20). This suggests that although NF-κB binding activity can be increased in response to IFN-γ this mechanism is not involved in the regulation of MCP-1 expression by the cytokine.

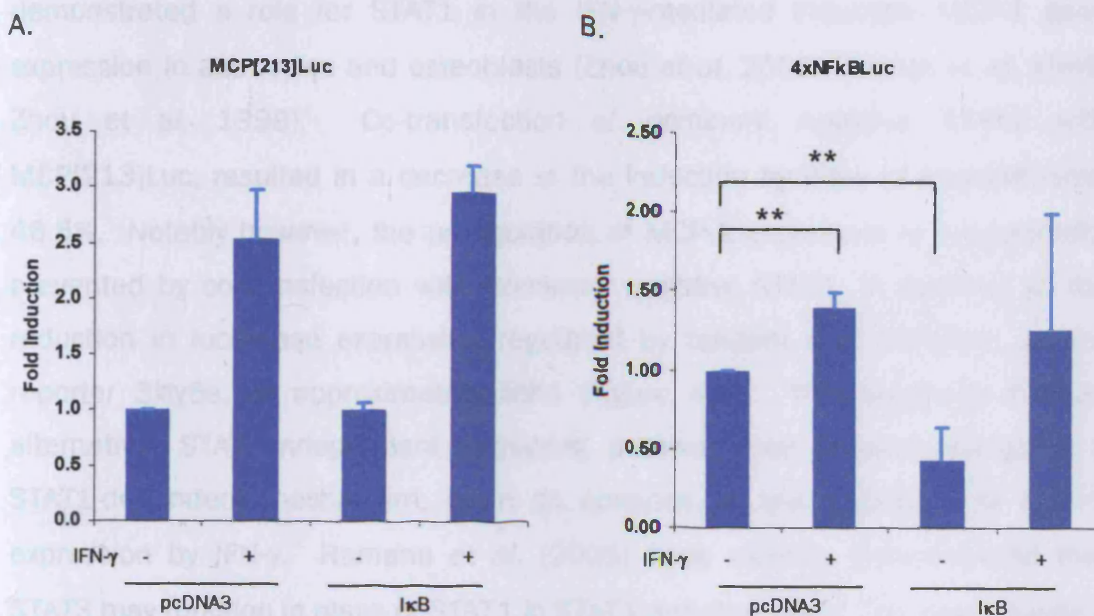


Figure 4.20 Role of NF-κB in the the induction of MCP-1 expression by IFN-γ

Hep3B cells were transfected with MCP[213]Luc (A) or 4xNF-κBLuc (B) and a plasmid construct expressing IκB or the empty vector pcDNA3. Transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration and presented as the mean fold induction (±SD) in response to IFN-γ, in relation to basal expression (assigned as 1), from three independent experiments. In the case of co-transfections for IκB and the 4xNF-κBLuc reporter, counts were normalised to the basal expression of 4xNF-κBLuc transfected with pcDNA3. **P<0.01.

4.5 DISCUSSION

Due to the importance of MCP-1 in the progression of atherosclerosis and the potential to uncover a novel PI3K-dependent pathway in IFN-γ-signalling, further studies were carried out of the regulation of MCP-1 expression by this cytokine. The action of PI3K either in cross-talk with classical JAK-STAT signalling or in STAT1-independent induction of MCP-1 expression by IFN-γ was of particular interest.

4.5.1 Role of STAT1 in the regulation of MCP-1 expression by IFN-γ

Despite findings by Gil *et al.* (2001) that MCP-1 expression is inducible by IFN-γ in both wild type and STAT1-deficient macrophages previous studies have demonstrated a role for STAT1 in the IFN-γ-mediated induction MCP-1 gene expression in astrocytes and osteoblasts (Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998). Co-transfection of dominant negative STAT1 with MCP[213]Luc, resulted in a decrease in the induction by IFN-γ of approximately 46.5%. Notably however, the upregulation of MCP-1 expression is only partially prevented by co-transfection with dominant negative STAT1, in contrast to the reduction in luciferase expression regulated by tandem GAS elements, in the reporter 3xly6e, of approximately 89% (Figure 4.4). This suggests that an alternative, STAT1-independent signalling pathway may operate alongside a STAT1-dependent mechanism, or in its absence, in the regulation of MCP-1 expression by IFN-γ. Ramana *et al.* (2005) have recently demonstrated that STAT3 may function in place of STAT1 in STAT1-deficient cells. This may provide a possible explanation for the finding of Gil *et al.* (2001) that the induction of MCP-1 expression by IFN-γ is unaffected in STAT1-null macrophages while in the cellular systems used in this study, and others, STAT1 has been shown to be required. While in STAT1 knock-out cells STAT3 may act in place of STAT1, a dominant negative inhibitor such as that used here may also sequester active STAT3 through heterodimerisation and hence attenuate the response to IFN-γ. However, we cannot exclude a potential role for alternative, STAT-independent mechanisms.

The activation of STAT1 in J774.2 macrophages, through the phosphorylation at Tyrosine 701 and Serine 727 in response to IFN-γ, was demonstrated to occur after 5min at both sites but not reach a maximal level until after 30min treatment in the case of Serine 727 phosphorylation (Figure 4.7). The phosphorylation of both sites after 3h stimulation with IFN-γ, is inhibited by incubation with the JAK2 inhibitor AG490 as expected. Notably, the basal level of STAT1 phosphorylation at Serine 727 was increased by AG490 treatment, suggesting that JAK2 may play a negative regulatory role in unstimulated cells (Figure 4.8).

Binding to a region of the human MCP-1 promoter containing a GAS element (-213) has been demonstrated previously by Zhou *et al.* (1998) to be inducible by IFN-γ in astrocytoma cells. EMSA analysis showed a similar response in J774.2 cells after 30min, declining after 3h treatment with IFN-γ (Figure 4.12). ChIP analysis confirmed that STAT1 binds to this region of the proximal MCP-1 promoter in the cellular environment and that this binding is increased by stimulation with IFN-γ for 30min (Figure 4.17). The transient nature of STAT1 binding is different from the prolonged induction of MCP-1 mRNA expression by IFN-γ. It is possible that the MCP-1 mRNA transcript, induced following short term IFN-γ treatment, is stable over this period of time. Alternatively following STAT1 binding to the MCP-1 promoter, a secondary mechanism may be activated that does not require the presence of STAT1.

4.5.2 Role of JAK1 and JAK2 in the regulation of MCP-1 experssion by IFN-γ

Transfection of dominant negative forms of JAK1 and JAK2 in Hep3B cells led to a decrease in the IFN-γ response of the MCP[213]Luc promoter of 75.8% and 92.6% respectively (Figure 4.4). This indicates that JAK2 is essential for the upregulation of MCP-1 by IFN-γ and that if two divergent pathways do exist, they must operate downstream of this kinase. In confirmation of this role, the JAK2 inhibitor AG490 also prevents the induction of both MCP-1 mRNA and protein expression in J774.2 cells by IFN-γ (Figure 4.2 and 4.3). The inhibition of JAK1 using a dominant negative construct appears to decrease the IFN-γ response to a lesser degree than JAK2. Some difference in the degree of inhibition of the

induction of expression from the construct 3xly6e, regulated by three tandem GAS elements, was also seen with dominant negative JAK1 and JAK2 (82% and 88.2% respectively) indicating that JAK2 may retain some ability to phosphorylate STAT1 in the absence of JAK1 kinase activity, or merely that the JAK2 mutant blocks kinase activity more efficiently than the mutant JAK1 (Figure 4.4). However, JAK2 may also have a STAT1-independent role that does not require the activation of JAK1.

4.5.3 Role of PI3K in the regulation of STAT1 in response to IFN-γ-stimulation

Several previous studies have implicated PI3K in the regulation of STAT1 activity and suggest that the kinase functions in the phosphorylation of STAT1 on Serine 727. These studies have shown that LY294002 has no effect on the phosphorylation of Tyrosine 701 in response to stimulation with IFN-γ, but that the phosphorylation of Serine 727 is inhibited in fibroblasts, epithelial and mesangial cells (Choudhury 2004, Higashi *et al.* 2003, Nguyen *et al.* 2001). In contrast Hwang *et al.* (2004) found that LY294002 inhibited the IFN-γ-mediated phosphorylation of STAT1 at Tyrosine 701 in microglial cells. The data shown here indicate that LY294002 affects the IFN-γ-induced phosphorylation of STAT1 at Serine 727 in macrophages at a concentration that inhibits mRNA expression. Co-transfection of a dominant negative mutant of PKB with the reporter construct 3xly6e reduces the stimulation of expression by IFN-γ by approximately 57%, confirming an effect on STAT1 activation.

4.5.4 Activation of PI3K in response to IFN-γ-stimulation potentially occurs through JAK activity

It has been suggested that phosphorylation of the IFN-γ receptor may provide a docking site for PI3K recruitment and previous studies have demonstrated functional interactions between the p85 subunit of PI3K and JAK1 and JAK2, inducible by various cytokines (Okugawa *et al.* 2003, Rane and Reddy 2000, Al-Shami and Naccache 1999, Oh *et al.* 1998, Yamauchi *et al.* 1998). The transfection of dominant negative JAK1 and JAK2 mutants with MCP[213]Luc

indicated that these kinases, in particular JAK2, are essential for the IFN-γ-mediated induction of MCP-1. The possibility that PI3K activity may be induced through activated JAKs was therefore investigated.

Dominant negative and inhibitor studies previously indicated that PKB is a likely downstream effector of PI3K in the IFN-γ-mediated induction of MCP-1 expression (Section 3.4). The phosphorylation and kinase activity of PKB were both found to be increased maximally in J774.2 cells by incubation with IFN-γ for 30min. This corresponds to a maximal increase in STAT1 phosphorylation at Serine 727 following 30min stimulation with IFN-γ. The activation of PKB in response to the cytokine was inhibited by pretreatment with both LY294002 and AG490. This suggests that PI3K and its downstream effector PKB are activated by IFN-γ through the action of JAK2.

Co-immunoprecipitation experiments showed a constitutive interaction between JAK2 and the PI3K p85 subunit that is not increased by IFN-γ treatment. It remains to be determined if this is a direct interaction or occurs through the IFN-γ receptor or another adaptor molecule. Al-Shami and Naccache (1999) have established that JAK2 is required for the phosphorylation of p85 in response to GM-CSF and showed that an interaction occurred between JAK2 and p85 dependent on an unidentified adaptor protein. Yamauchi *et al.* (1998) demonstrated that JAK2 binds to p85 in response to GH and PRL through IRS-1. The constitutive nature of the interaction found here suggests that while PI3K may be constitutively recruited to JAKs and/or the IFN-γ receptor, it is only activated when the kinases are activated by IFN-γ. Future experiments will seek to determine whether a similar interaction is seen between p85 and JAK1 and how this coincides with the activation of JAKs by IFN-γ and the phosphorylation of the IFN-γ receptor. It is also worth noting that Nguyen *et al.* (2001) demonstrated that the activation of PI3K occurred in both JAK1 and JAK2-null cells although there may be a degree of functional redundancy involved.

4.5.5 Role of CK2 in the regulation of STAT1 in response to IFN-γ-stimulation

Previous studies have linked CK2 to the regulation of the activity of downstream effectors of PI3K including PTEN and PKB (Di Maira *et al.* 2005, Torres and Pulido 2001). However in the cellular systems used in this work the activation of CK2 appears to occur in a mechanism that is entirely independent of PI3K activation and may be independent of JAK signalling. IFN-γ-induced activation of CK2 kinase activity is not inhibited by AG490 or LY294002 (Evans, S., personal communication), both of which attenuate the activation of PKB in response to the cytokine. On the other hand the activation of PKB in response to IFN-γ is unaffected by the presence of apigenin (Figure 4.10).

To further investigate the role of CK2 in the IFN-γ response, co-transfection and western blot experiments were carried out to determine a potential link between CK2 and the activation of STAT1. Co-transfection of dominant negative CK2 with the luciferase reporter construct 3xly6e indicated that CK2 does, to some extent, regulate the promoter binding activity of STAT1, IFN-γ inducible expression being reduced by approximately 39.4% (Figure 4.5). Western blot experiments indicated that CK2 does not affect the phosphorylation of STAT1 at Tyrosine 701, as has previously been demonstrated in fibroblasts (Higashi *et al.* 2003), but may be involved in the phosphorylation of Serine 727, as the IFN-γ-inducible phosphorylation of this residue is prevented by treatment with apigenin (Figure 4.8). Co-immunoprecipitation experiments suggest that STAT1 is not directly phosphorylated by CK2 as there is no interaction between the two molecules either in control or IFN-γ stimulated cells (Figure 4.18). This suggests that a downstream STAT1-kinase is potentially regulated through both CK2 and PKB.

Table 4.1 summarises the effect of the dominant negative constructs used in this study on the activation of the MCP-1 promoter by IFN-γ and the effect on the activity of a GAS-driven reporter.

Table 4.1 Relative inhibition of IFN-γ-stimulated MCP-1 and GAS regulated promoter activity by dominant negative constructs

	% DECREASE	
TARGET	MCP[213]LUC	3xly6e
STAT1 (Tyr701)	46.5	89.0
STAT1 (Ser727)	36.5	42.8
JAK1	75.8	82.0
JAK2	92.6	88.2
CK2	45.1 (basal)	39.4
PKB	46.9	57.0

4.5.6 Role of Sp1 in the regulation of MCP-1 expression by IFN-γ

The transcription factor Sp1 is usually considered to have a role in constitutive gene expression although inducible regulation has also been demonstrated. Sp1 binds to a consensus promoter element known as a GC box, often found near binding sites for other transcription factors, suggesting that they may act to regulate gene expression in conjunction with other proteins (Section 1.8.2.1). Sp1 has been shown in our laboratory to be involved in the downregulation of LPL by IFN-γ, whereby CK2 phosphorylates Sp1 leading to a decrease in promoter binding activity (Hughes *et al.* 2002, Armstrong *et al.* 1997, Evans, S., personal communication). An interaction has been shown by work in our laboratory, to exist between CK2 subunits and Sp1, that is inducible by IFN-γ for the α and α' subunits and constitituve for CK2β (Evans, S., personal communication) and is reproduced in Figure 4.18. In the regulation of MCP-1 expression by IFN-γ, Sp1 has previously been found to bind constitutively to the human MCP-1 promoter in astrocytoma cells, while STAT1 binding is increased by the cytokine. Mutation of the GC box in the human promoter also decreases basal activity of a luciferase reporter construct (Zhou *et al.* 1998). Constitutive binding of Sp1 to the GC box of the proximal promoter of the murine MCP-1 gene was demonstrated by EMSA in J774.2 macrophages (Figure 4.11) and by ChIP is U937 cells (Figure 4.17). It is possible to conceive a mechanism in which the phosphorylation of Sp1 by CK2 may be involved in the constitutive expression of MCP-1, shown to be decreased

by co-transfection with a dominant negative inhibitor of CK2. This may be mediated by an interaction with CK2β that is present in both control and IFN-γ treated cells.

A study by Look *et al.* (1995) into the regulation of the ICAM-1 gene proposes that there is an IFN-γ-inducible interaction between STAT1 and Sp1, by which they work synergistically in the upregulation of ICAM-1 expression in human epithelial cells. Such an interaction was not found in a similar experiment by Zhou *et al.* (1998) in relation to the IFN-γ-mediated regulation of MCP-1 in astrocytes. However, the co-immunoprecipitations described in this report have found that there is a marked increase in the binding of Sp1 and STAT1 by IFN-γ (Figure 4.18). The role of this interaction in the transcriptional regulation of gene expression is unclear. Sp1 may have a function in the recruitment of STAT1 to the GAS site of the MCP-1 promoter or the binding of the two transcription factors may regulate the DNA conformation of the promoter region. The phosphorylation of Sp1 by CK2 may also have role in mediating this interaction as may the phosphorylation of STAT1 at Serine 727 downstram of both CK2 and PI3K.

4.5.7 Role of other transcription factors in the regulation of MCP-1 expression by IFN-γ

Figure 3.11 illustrates the presence of several putative *cis*-acting regulatory elements in the MCP-1 promoter for transcription factors including Sp1, STAT1, NF-κB, AP-1 and C/EBP-β. Previous studies have indicated a role for all of these factors in the regulation of MCP-1 expression in response to cytokine stimulation (Table 1.7). Competition studies in EMSA suggest that the AP-1 and NF-κB sites that overlap the GC-box and GAS element respectively are not involved in the regulation of MCP-1 expression. The role of C/EBP-β binding and binding to a second κB site in the human MCP-1 promoter in the response to IFN-γ-stimulation had not been studied. Transcriptional activation of both C/EBP-β and NF-κB has also been linked to IFN-γ-signalling and may be regulated by both PI3K and CK2 (Section 1.82.1). The activity of promoters regulated by tandem C/EBP-β and NF-κB binding elements (4xC/EBPLuc and 4xNFκBLuc respectively) was upregulated

by IFN-γ. However the overexpression of the endogenous inhibitors of C/EBP-β and NF-κB activity, LIP and IκB respectively, in co-transfection studies with MCP[213]Luc indicated that neither of these factors is necessary for the regulation of MCP-1 promoter activation in response to IFN-γ. The efficacy of these inhibitors was confirmed by co-transfection with reporter constructs regulated by multimers of C/EBP-β and NF-κB elements, the activity of which was reduced by the presence of LIP and IκB respectively. *Cis*-acting elements shown to bind these factors are also present in the distal region of the MCP-1 promoter however and the possibility remains that they may be able to enhance the IFN-γ-inducible expression of the MCP-1 gene through these sites (Figure 3.11).

CHAPTER FIVE:

GENE-SPECIFIC FUNCTION OF PI3K IN IFN-γ SIGNALLING

CHAPTER 5. GENE-SPECIFIC FUNCTION OF PI3K IN IFN-γ SIGNALLING

5.1 INTRODUCTION

The identification of PI3K as a signalling mediator in the regulation of MCP-1 gene expression by IFN-γ, but not in the regulation of SOCS-1 or ICER (Figure 3.5), raised the possibility that a specific subset of ISGs are dependent on the activation of PI3K. If such a gene-specific pathway were to exist it may provide targets for the selective inhibiton of certain IFN-γ responses in the treatment of atherosclerosis.

5.1.1 Experimental strategy

In order to investigate the gene-specific role of PI3K in IFN-γ signalling further, a strategy was developed to study the regulation of groups of genes by the cytokine by RT-PCR analysis and using gene array technology. Experiments were carried out in J774.2 macrophages. Due to the difficulties with the efficient transfection of this cell line, the pharmacological agent LY294002 was used, as opposed to dominant negative mutants, in order to assess the requirement for PI3K. The role of CK2 and JAK2 in the regulation of selected genes by IFN-γ was also to be investigated, using the inhibitors apigenin and AG490 respectively, in order to determine whether the regulatory mechanisms involved are comparable to those responsible for the induction of MCP-1. Additionally, key experiments were performed in endothelial cells to assess the possibility that findings might be relevant to IFN-γ-mediated signalling in other cell types of the atherosclerotic plaque. The overall experimental strategy for work presented in this chapter is illustrated in Figure 5.1.

5.1.2 Regulation of chemokine gene expression by IFN-γ

Chemokines are important inflammatory mediators in the progression of atherosclerosis, discussed in detail in Section 1.5. Considering this role, and in order to investigate the possibility that a common mechanism may regulate a number of chemokines in addition to MCP-1, this group of genes was selected for

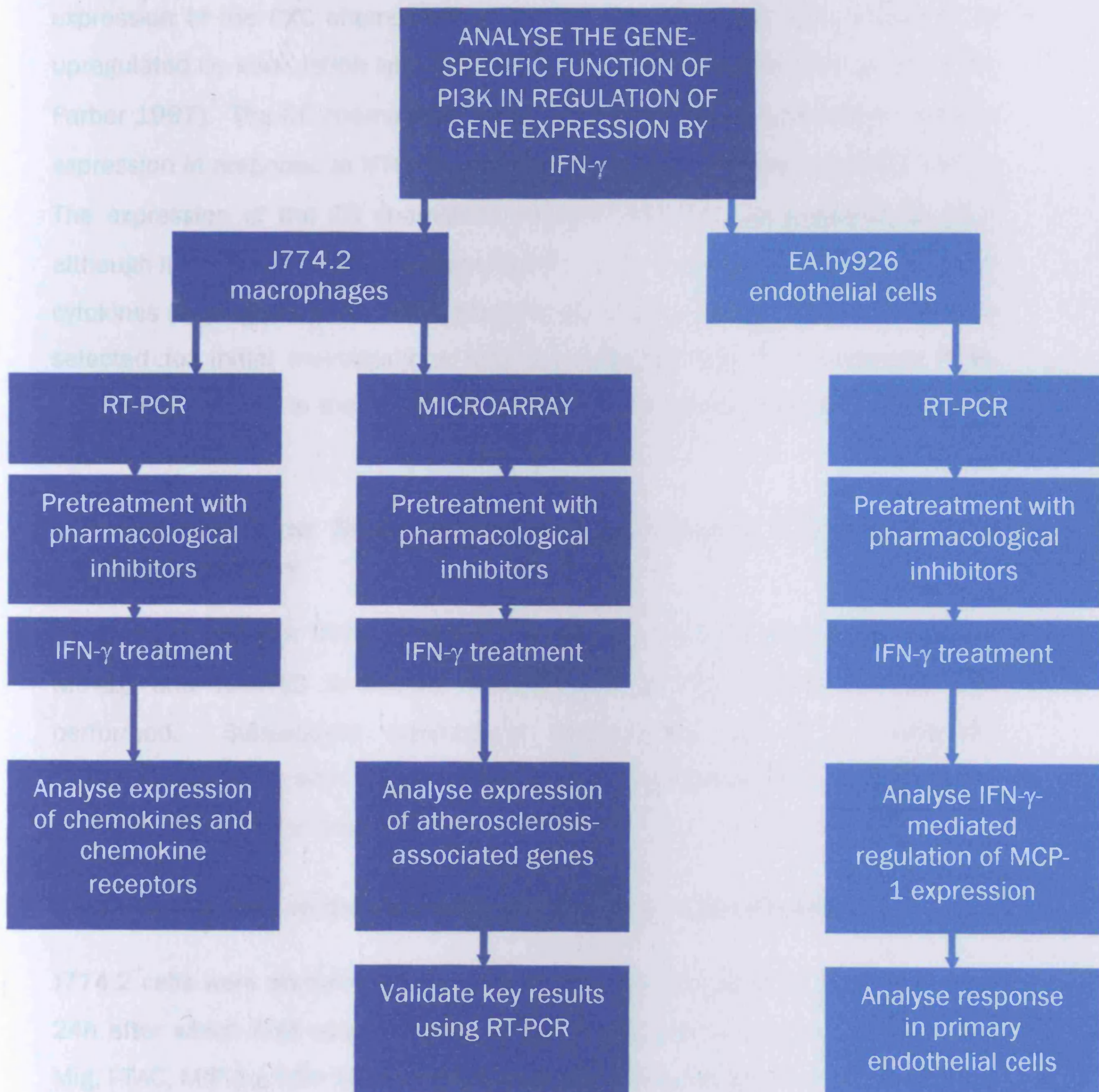


Figure 5.1 Experimental strategy

In order to extend the previous findings with regards to the role of PI3K in IFN-γ signalling, a broad strategy to identify a potential requirement for PI3K in the regulation of other atherosclerosis-associated genes by the cytokine was developed, involving the use of pharmacological inhibitors coupled with RT-PCR and microarray analyses. The investigation of the role of PI3K in the regulation of MCP-1 expression by IFN-γ was also extended to studies in endothelial cells.

further study of IFN-γ-mediated mechanisms of transcriptional regulation. The expression of the CXC chemokines IP-10, Mig and I-TAC has been shown to be upregulated by stimulation with IFN-γ in a variety of cell types (Cole *et al.* 1998, Farber 1997). The CC chemokines MIP-1α and -1β have also exhibited inducible expression in response to IFN-γ in macrophage cell lines (Martin and Dorf 1991). The expression of the CC chemokine RANTES may also be regulated by IFN-γ although it has more commonly been found to act in synergy with TNF-α and other cytokines (Wakugawa *et al.* 2001, Marfaing-Koka *et al.* 1995). These genes were selected for initial investigations into the potential role of a common PI3K-dependent pathway in the IFN-γ-induced expression of these genes in addition to MCP-1.

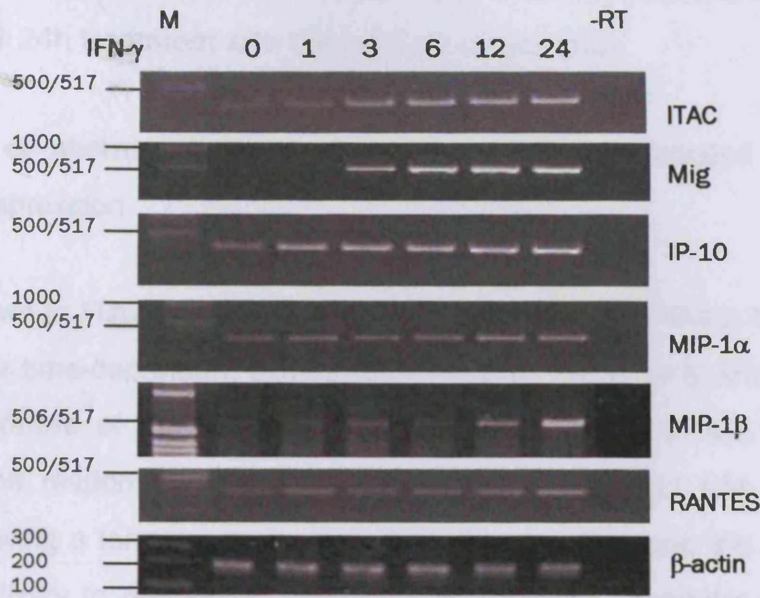
5.2 PI3K-DEPENDENT SIGNALLING IN THE REGULATION OF CHEMOKINE GENE EXPRESSION BY IFN-γ

To establish whether IFN-γ affects the expression of IP-10, Mig, I-TAC, MIP-1α, MIP-1β and RANTES in J774.2 macrophages a time course analysis was performed. Subsequent experiments involved the use of the inhibitors LY294002, apigenin and AG490 to identify a potential role of PI3K, CK2 and JAK2 in mediating the IFN-γ response.

5.2.1 Effect of IFN-γ on the expression of chemokines in macrophages

J774.2 cells were stimulated with murine IFN-γ for periods of 0, 1, 3, 6, 12 and 24h after which RNA was extracted. RT-PCR was carried out for murine IP-10, Mig, I-TAC, MIP-1α, MIP-1β, RANTES and β-actin using the primers and conditions detailed in Table 2.5, generating products of the appropriate size. Figure 5.2 summarises the change in mRNA levels for each species in response to IFN-γ, over the duration of the time course. Figure 5.2A illustrates the PCR products fractionated by agarose gel electrophoresis. The band densities were normalised to the expression of β-actin and the fold induction in comparison to the mRNA expression at 0h (assigned as 1) was plotted in relation to time (h) (Figure 5.2B). Gene expression levels for the trio of CXC chemokines IP-10, Mig and I-TAC were highest following stimulation with IFN-γ for between 3-6h (Figure 5.2). On the

A.



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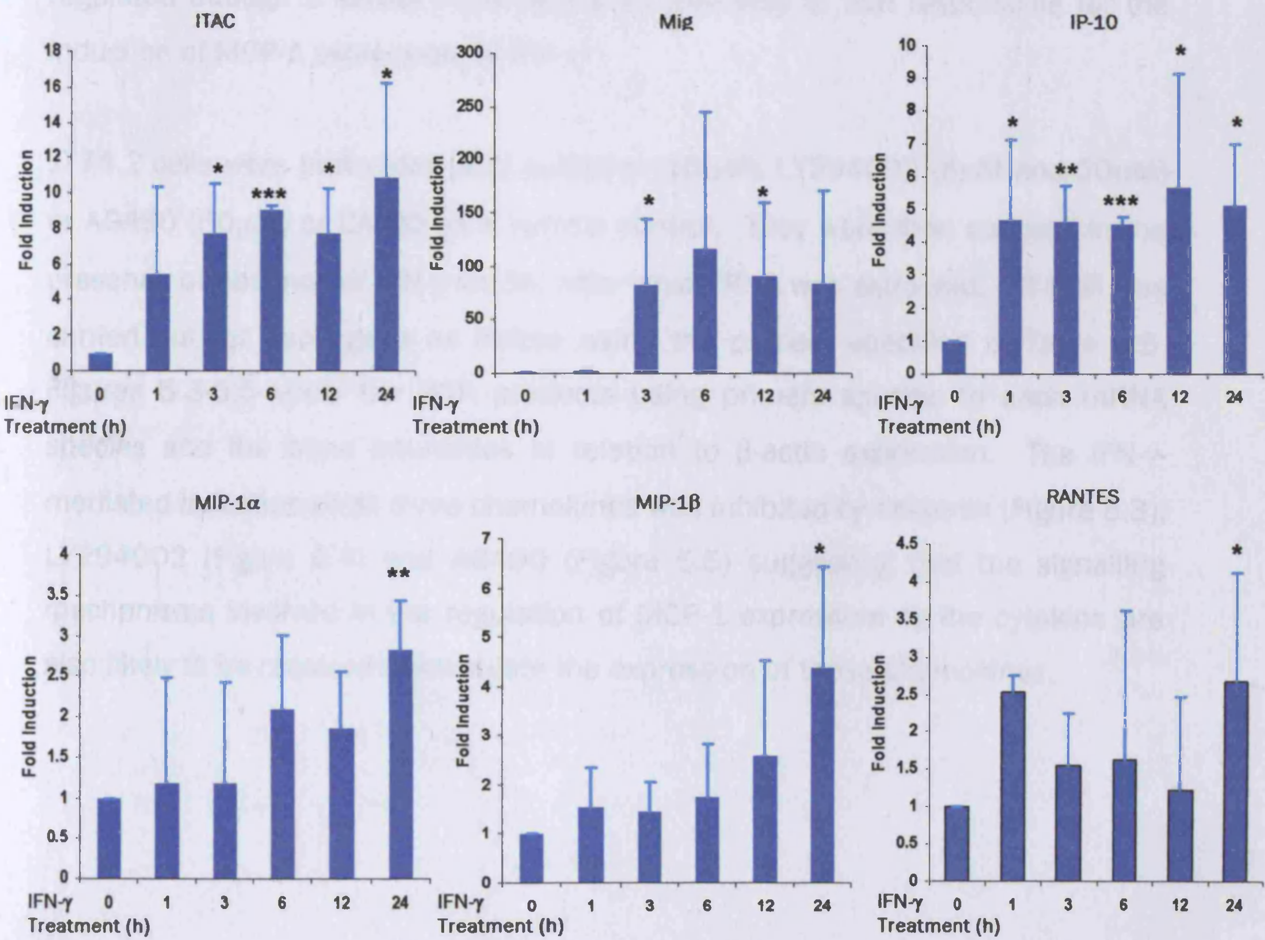


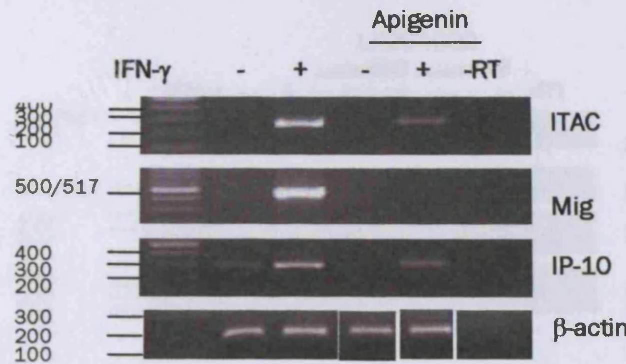
other hand, expression of the CC chemokines MIP-1α, MIP-1β and RANTES were maximal after 24h treatment with the cytokine (Figure 5.2).

5.2.2 Effect of pharmacological inhibitors on the IFN-γ-mediated induction of chemokine expression

The data shown in Figure 5.2 indicated that the chemokines IP-10, Mig and I-TAC have a similar time-dependent expression profile, in response to IFN-γ, to MCP-1. They are therefore of the most interest for a comparison of the mechanisms involved in this response. The expression of MIP-1α, MIP-1β and RANTES was induced following a longer period of incubation with IFN-γ and the mechanisms involved are likely to require *de novo* protein synthesis. Inhibitor studies were subsequently carried out in order to determine whether IP-10, Mig and I-TAC are regulated through a similar PI3K-dependent pathway to that responsible for the induction of MCP-1 expression by IFN-γ.

J774.2 cells were pretreated with apigenin (10μM), LY294002 (5μM and 20μM) or AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h, after which RNA was extracted. RT-PCR was carried out for each gene as before using the primers specified in Table 2.5. Figures 5.3-5.5 show the PCR products using primers specific to each mRNA species and the band intensities in relation to β-actin expression. The IFN-γ-mediated induction of all three chemokines was inhibited by apigenin (Figure 5.3), LY294002 (Figure 5.4) and AG490 (Figure 5.5) suggesting that the signalling mechanisms involved in the regulation of MCP-1 expression by the cytokine are also likely to be required to stimulate the expression of these chemokines.

A.



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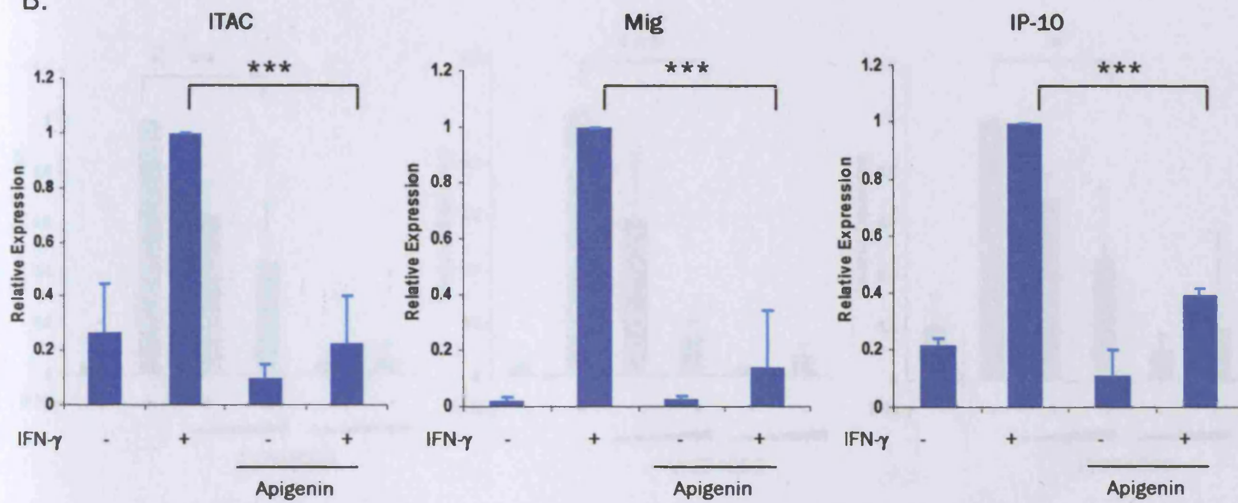
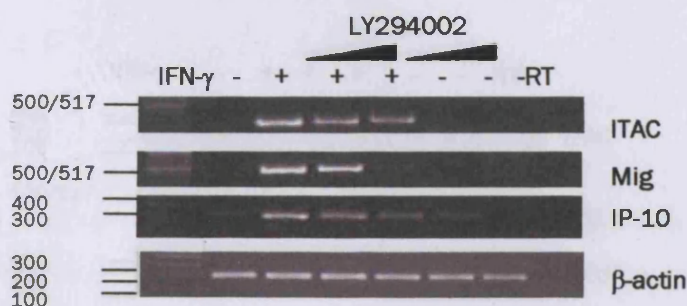


Figure 5.3 Effect of the CK2 inhibitor apigenin on the induction of chemokine expression by IFN-γ

J774.2 macrophages were pretreated with apigenin (10μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against: ITAC; Mig; IP-10; β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expresssion of β-actin as determined by densitometric analysis from at least three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). ***P<0.001.

A.



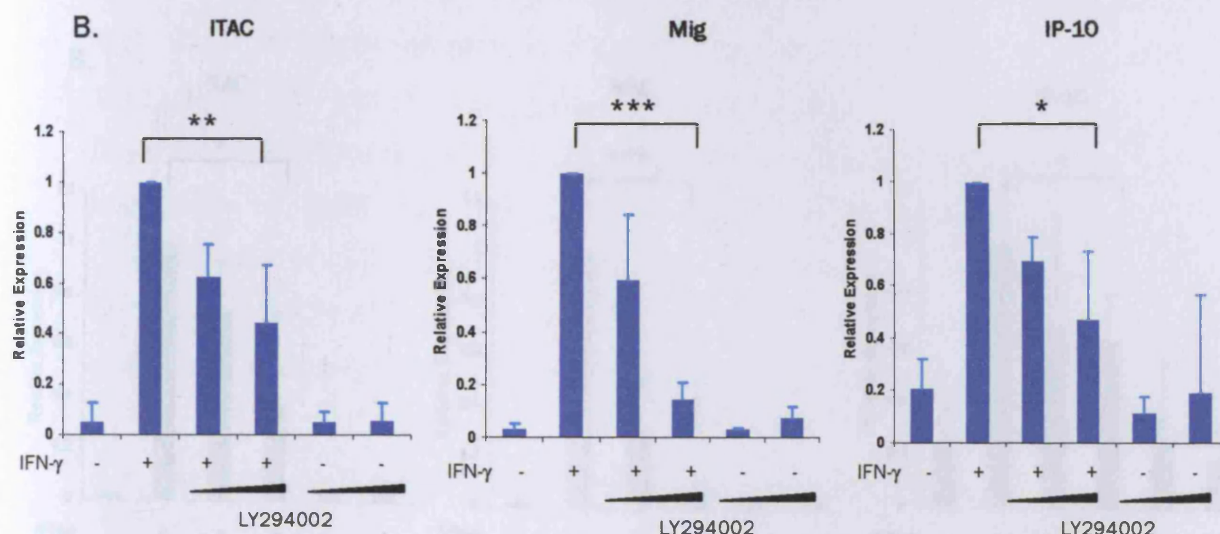


Figure 5.4 Effect of the PI3K inhibitor LY294002 on the induction of chemokine expression by IFN-γ

J774.2 macrophages were pretreated with LY294002 (5μM and 20μM)or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against: ITAC; Mig; IP-10; β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expresssion of β-actin as determined by densitometric analysis from at least three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). *P<0.05; **P<0.01; ***P<0.001.

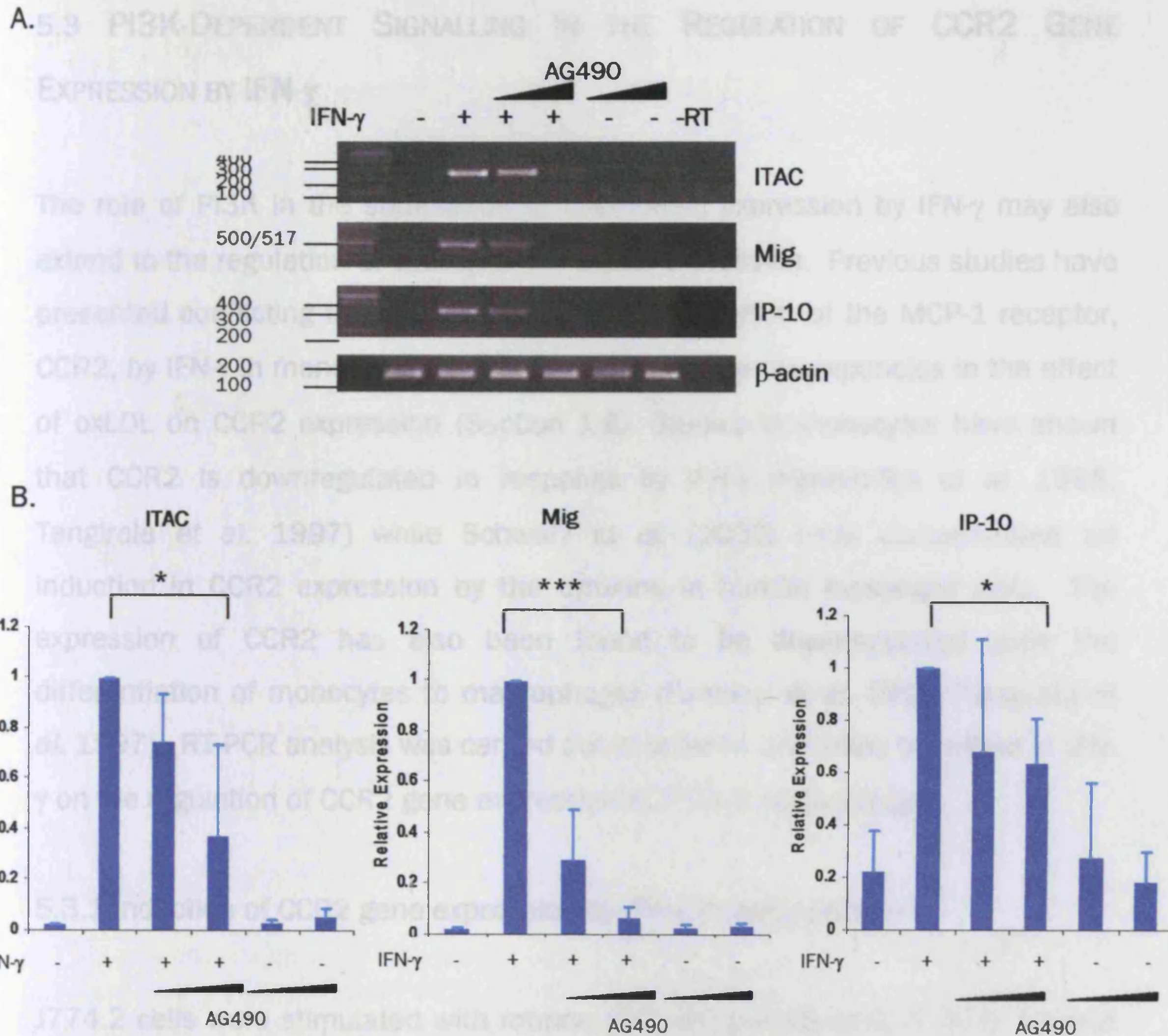


Figure 5.5 Effect of the JAK2 inhibitor AG490 on the induction of chemokine expression by IFN-γ

J774.2 macrophages were pretreated with AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against: ITAC; Mig; IP-10; β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expresssion of β-actin as determined by densitometric analysis from at least three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). *P<0.05; ***P<0.001.

5.3 PI3K-DEPENDENT SIGNALLING IN THE REGULATION OF CCR2 GENE EXPRESSION BY IFN-γ

The role of PI3K in the stimulation of chemokine expression by IFN-γ may also extend to the regulation of chemokine receptor expression. Previous studies have presented conflicting data with regards to the regulation of the MCP-1 receptor, CCR2, by IFN-γ in monocytes, corresponding to similar discrepancies in the effect of oxLDL on CCR2 expression (Section 1.6). Studies in monocytes have shown that CCR2 is downregulated in response to IFN-γ (Penton-Roi *et al.* 1998, Tangirala *et al.* 1997) while Schwarz *et al.* (2002) have demonstrated an induction in CCR2 expression by the cytokine in human mesangial cells. The expression of CCR2 has also been found to be downregulated upon the differentiation of monocytes to macrophages (Fantuzzi *et al.* 1999, Tangirala *et al.* 1997). RT-PCR analysis was carried out in order to determine the effect of IFN-γ on the regulation of CCR2 gene expression in J774.2 macrophages.

5.3.1 Induction of CCR2 gene expression by IFN-γ in macrophages

J774.2 cells were stimulated with murine IFN-γ for periods of 0, 1, 3, 6, 12 and 24h after which RNA was extracted. RT-PCR was carried out for murine CCR2 and β-actin using the primers and conditions detailed in Table 2.5, generating products of the appropriate size. Figure 5.6 illustrates the PCR products fractionated by agarose gel electrophoresis (Figure 5.6A). The band densities were normalised to the expression β-actin and the fold induction in comparison to the mRNA expression at 0h (assigned as 1) was plotted in relation to time (h) (Figure 5.6B). IFN-γ was found to induce the expression of CCR2 mRNA, maximal levels being reached after 3-6h cytokine stimulation.

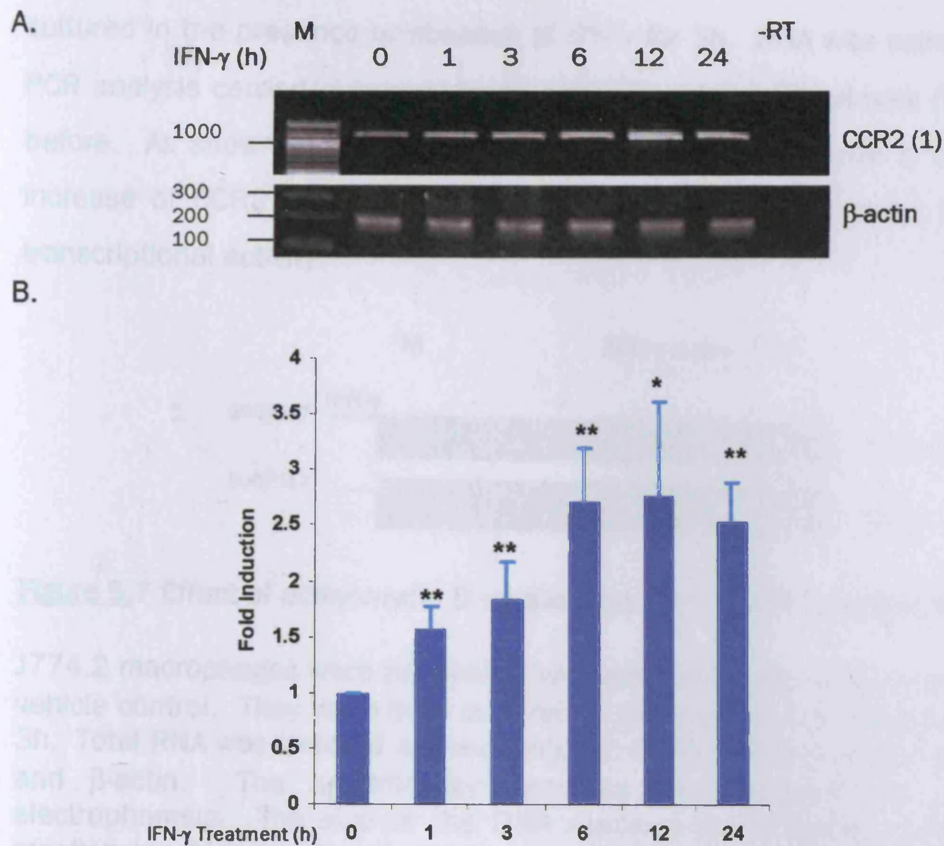


Figure 5.6 Induction of CCR2 mRNA expression by IFN-γ

J774.2 macrophages were exposed to IFN-γ for the indicated time. Total RNA was isolated and subjected to RT-PCR using primers against CCR2 and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The position of the appropriate product and the size of the DNA markers are indicated. Panel B shows the fold induction (mean ±SD) in the expression of the appropriate gene normalised to the expression of β-actin as determined by densitometric analysis from three independent experimental series. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using untreated RNA). *$P<0.05$; **$P<0.001$.

5.3.2 IFN-γ-mediated upregulation of CCR2 mRNA levels in macrophages is dependent on transcriptional activity but not mRNA stability

Notably, the increase of CCR2 mRNA levels was not as dramatic as for MCP-1 and the CXC chemokines, thereby suggesting the possibility that it may be mediated by a more moderate effect of IFN-γ on mRNA stability and not transcriptional activation. In order to determine whether this was the case, the pharmacological inhibitor of transcription, actinomycin D was used. J774.2 cells were pretreated with actinomycin D (5μg/ml) or DMSO as a vehicle control. They were then

cultured in the presence or absence of IFN-γ for 3h. RNA was extracted and RT-PCR analysis carried out using murine CCR2 and β-actin primers (Table 2.5) as before. As shown in Figure 5.7, the presence of actinomycin D prevented the increase of CCR2 mRNA by IFN-γ, demonstrating a requirement for increased transcriptional activity.

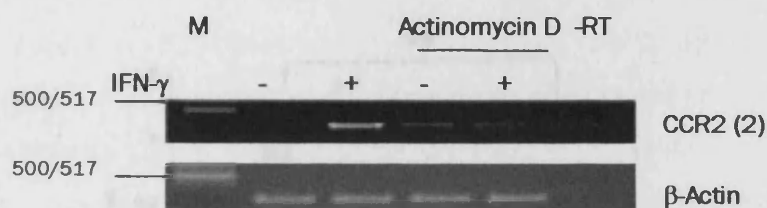


Figure 5.7 Effect of actinomycin D on the induction of MCP-1 mRNA levels by IFN-γ

J774.2 macrophages were pretreated with actinomycin D (5μg/ml) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against CCR2 and β-actin. The amplification products were analysed by agarose gel electrophoresis. The size of the DNA markers are indicated. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). Results shown are representative of two independent experiments.

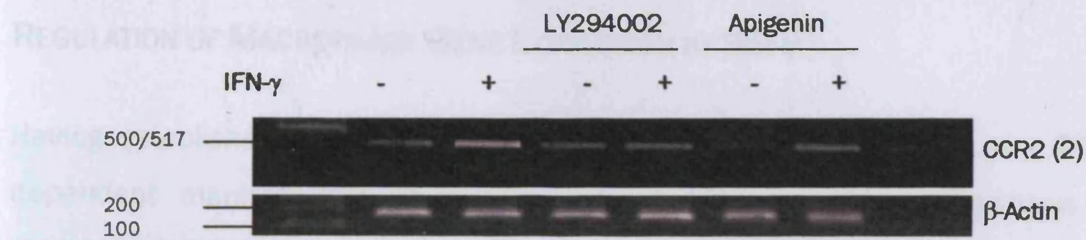
5.3.3 Effect of pharmacological inhibitors on the IFN-γ-mediated induction of CCR2 gene expression

The action of the pharmacological inhibitors LY294002, apigenin and AG490 on the IFN-γ-mediated induction of CCR2 gene expression was analysed in order to determine whether a similar PI3K-dependent pathway may be involved in the regulation of both MCP-1 and its receptor by the cytokine. J774.2 cells were pretreated with LY294002 (20μM), apigenin (20μM), AG490 (50μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which RNA was extracted. RT-PCR was carried out using primers specifying for CCR2 and β-actin as before. Figure 5.8 shows the PCR products resolved by agarose gel electrophoresis and the band intensities in relation to β-actin expression. The IFN-γ-mediated induction of CCR2 expression was inhibited by LY294002, apigenin (Figure 5.8A) and AG490 (Figure 5.8B) suggesting that the signalling mechanisms involved in the regulation of MCP-1 expression by the cytokine may also be required to stimulate the expression of the receptor CCR2.

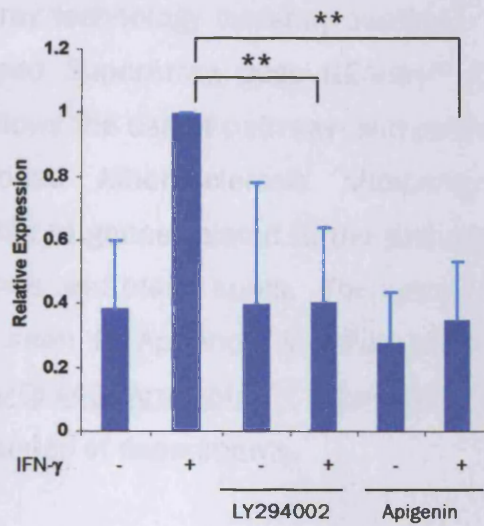
Figure 5.8 Effect of inhibitors on the induction of CCR2 expression by IFN-γ

J774.2 macrophages were pretreated with LY294002 (20μM), apigenin (20μM) (A), AG490 (50μM) (B) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against CCR2 and β-actin. The amplification products were analysed by agarose gel electrophoresis (A.i and B.ii). The size of the DNA markers are indicated. Panel A.ii and B.ii shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis from three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). **$P<0.01$; ***$P<0.001$.

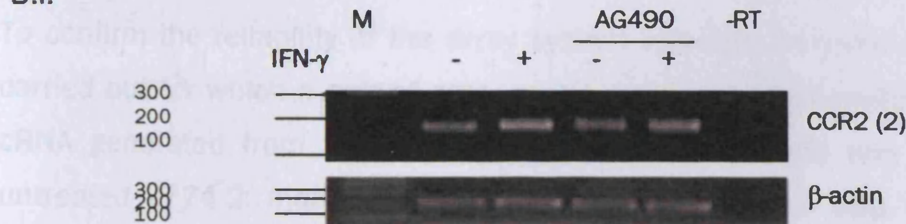
A.i.



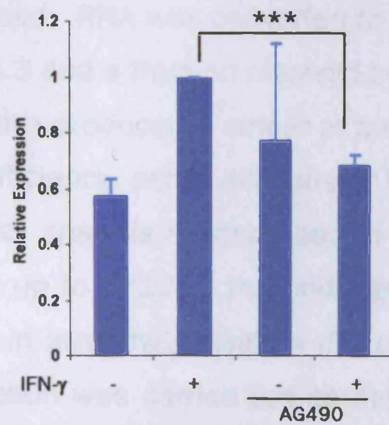
ii.



B.i.



ii.

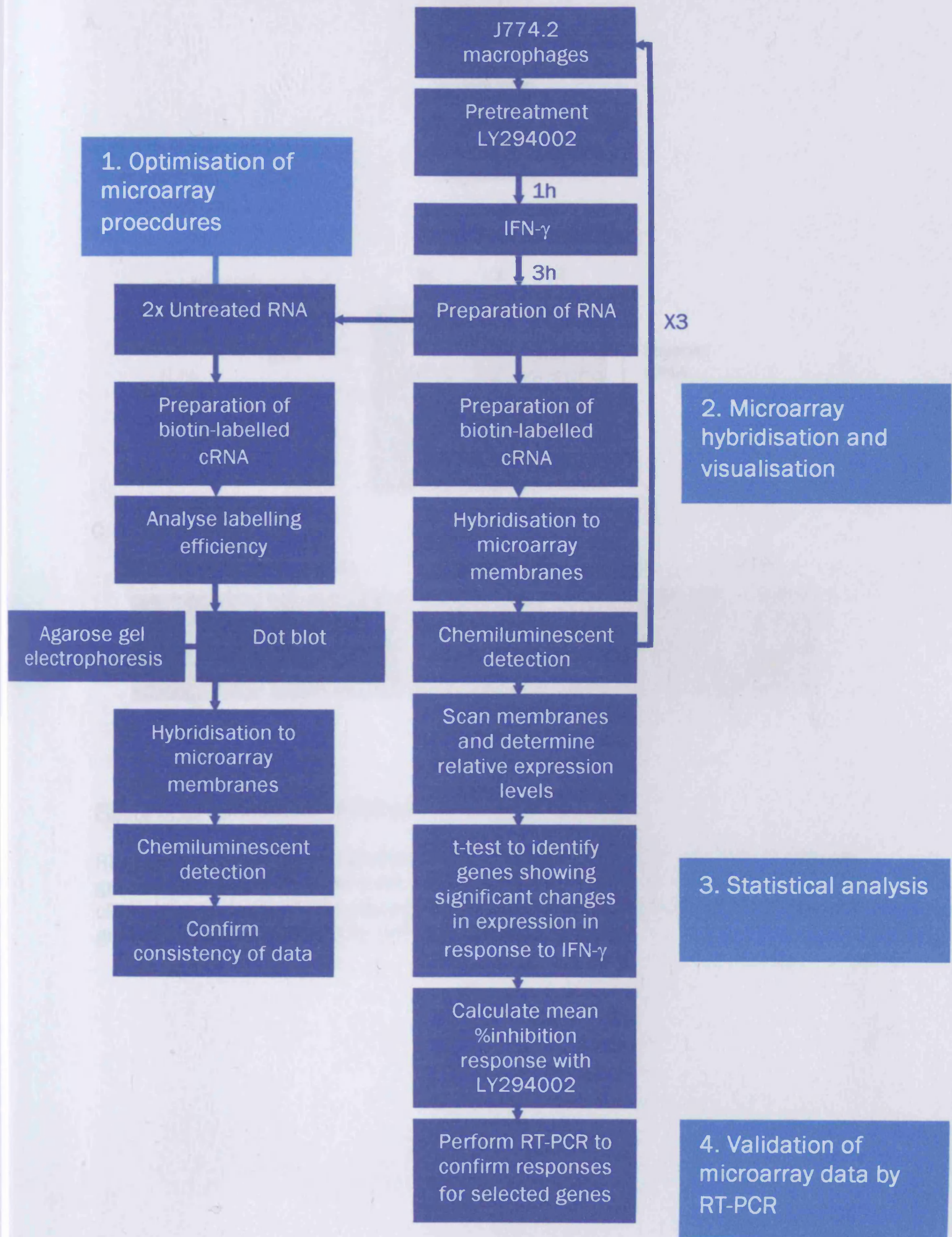


5.4 MICROARRAY ANALYSIS OF THE ROLE OF PI3K-DEPENDENT SIGNALLING IN THE REGULATION OF MACROPHAGE GENE EXPRESSION BY IFN-γ

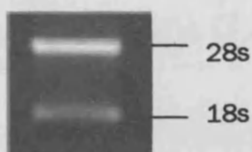
Having established a subset of genes that are regulated by IFN-γ in a PI3K-dependent manner, this study was extended to the global regulation of macrophage gene expression through the use of microarray analysis. Following a review of the gene array technology currently available, the system selected was the Nylon matrix based SuperArray Oligo GEArray™ DNA Microarray (Section 2.8.1). This system allows the use of pathway- and pathology-specific arrays. The Oligo GEArray™ Mouse Atherosclerosis Microarray used contains 113 oligonucleotides specific to genes related to the pathogenesis of atherosclerosis as well as control genes and blank spots. The complete list and grid layout of these genes can be seen in Appendix V. Full information can be found at http://superarray.com/OligoGEArray.php. Figure 5.9 details the experimental strategy used for this series of experiments.

5.4.1 Optimisation of microarray procedures

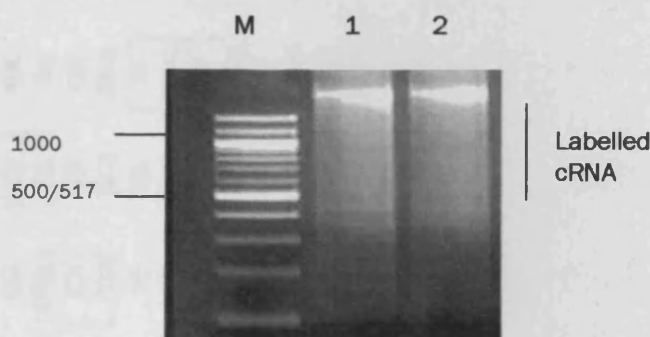
To confirm the reliability of the array system selected, an initial experiment was carried out for which a pair of array membranes were each probed with labelled cRNA generated from the same RNA sample. Total RNA was prepared from untreated J774.2 macrophages incorporating a DNaseI step into the usual protocol to minimise gDNA contamination (Section 2.5.2). Figure 5.10A illustrates the quality of the RNA used. RNA was converted to a labelled sample of cRNA as described in Section 2.8.3 and a fraction resolved by agarose electrophoresis. As shown in Figure 5.10B this produced a smear of between 400-1500bp. In order to assay the labelling efficiency, serial dilutions of labelled cRNA were prepared and subjected to dot blot analysis as described in Section 2.8.4. A signal was observed at dilutions of up to 1/1280 that indicates that labelled cRNA is of a sufficient quality to use in array hybridisation (Figure 5.10C). Hybridisation and chemiluminescent detection was carried out as detailed in Section 2.8.5-2.8.6. Both membranes displayed a comparable pattern of gene expression. The pattern of gene expression produced using an initial sample of RNA from untreated J774.2 macrophages is shown in Figure 5.11.


J774.2 macrophages
Pretreatment LY294002
1h
IFN-γ
3h
Preparation of RNA
X3
1. Optimisation of microarray proecdures
2x Untreated RNA
Preparation of biotin-labelled cRNA
Analyse labelling efficiency
Agarose gel electrophoresis
Dot blot
Hybridisation to microarray membranes
Chemiluminescent detection
Confirm consistency of data
Preparation of biotin-labelled cRNA
Hybridisation to microarray membranes
Chemiluminescent detection
2. Microarray hybridisation and visualisation
Scan membranes and determine relative expression levels
t-test to identify genes showing significant changes in expression in response to IFN-γ
3. Statistical analysis
Calculate mean %inhibition response with LY294002
Perform RT-PCR to confirm responses for selected genes
4. Validation of microarray data by RT-PCR

A.



B.



C.

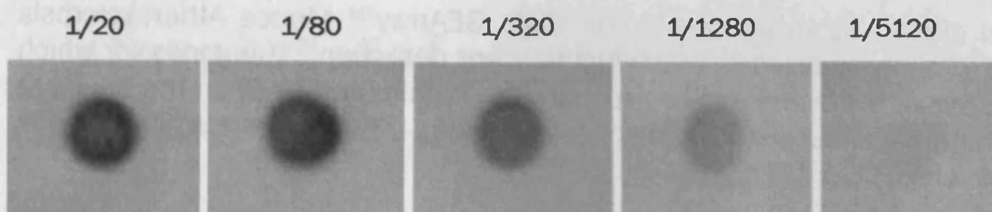
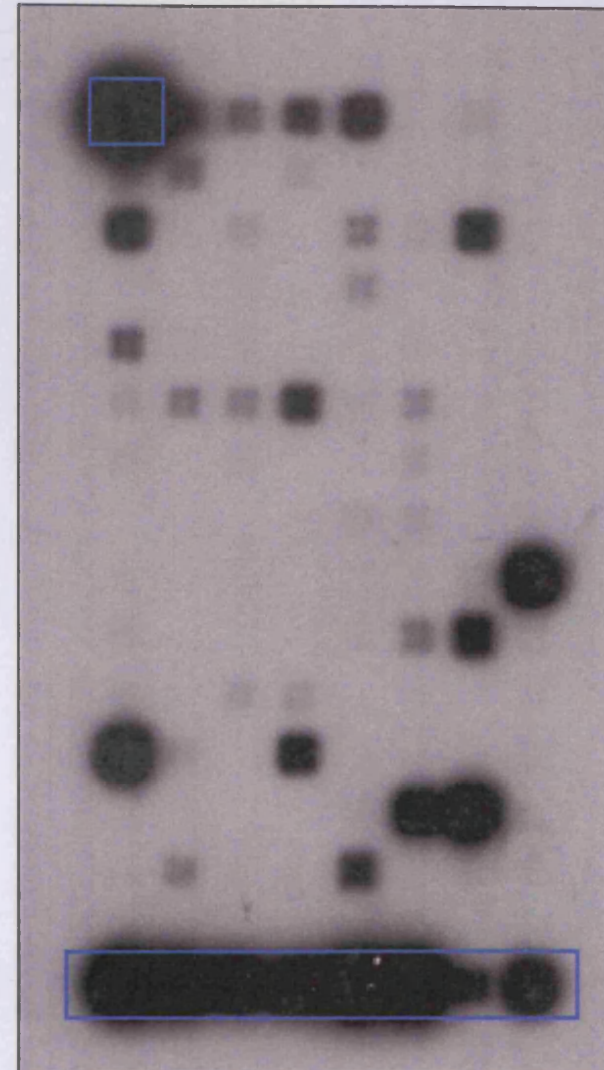


Figure 5.10 Optimisation of microarray procedures

RNA was extracted from untreated J774.2 macrophages and resolved by agarose gel electrophoresis to determine the integrity (A) and converted to biotin-labelled cRNA. The success of this procedure was assessed by resolving cRNA by agarose gel electrophoresis (B) and by dot blot analysis (C).

Gapdh 1	Abca1 2	Ace 3	Adfp 4	Apoa1 5	Apoa2 6	Apoa4 7	Apob 8
Apoe 9	Bax 10	Bcl2 11	Bcl2a1a 12	Bcl2l1 13	Bid 14	Birc3 15	Ccl11 16
Ccl2 17	Ccl20 18	Ccl5 19	Ccr1 20	Ccr2 21	Cd36 22	Cd44 23	Cdh5 24
Cflar 25	Col3a1 26	Csf1 27	Csf2 28	Csf3 29	Ctgf 30	Cxcl1 31	Hbegf 32
Egr1 33	Eln 34	Eng 35	F7 36	Fabp3 37	Fga 38	Fgb 39	Fgf2 40
Fn1 41	Icam1 42	Icam2 43	Ifnar2 44	Ifng 45	Il10 46	Il13 47	Il1a 48
Il1b 49	Il1r1 50	Il1r2 51	Il1rl1 52	Il2 53	Il3 54	Il4 55	Il5 56
Il6 57	Il7 58	Itga2 59	Itga5 60	Itgax 61	Itgb2 62	Itgb3 63	Itgb5 64
Itgb7 65	Kdr 66	Klf2 67	Lama1 68	Lcat 69	Ldlr 70	Lif 71	Lpl 72
Lypla1 73	Mmp13 74	Mmp1a 75	Mmp3 76	Mmp9 77	Msr1 78	Nfkb1 79	Npy 80
Nr1h3 81	Olr1 82	Pdgfa 83	Pdgfb 84	Pdgfrb 85	Ppara 86	Ppard 87	Pparg 88
Ptgs1 89	Ptgs2 90	Rxra 91	Scarb1 92	Sele 93	Sell 94	Selp 95	Selpl 96
Serpinb2 97	Serpine1 98	Smn 99	Soat2 100	Sod1 101	Sod2 102	Spp1 103	Tgfb1 104
Tgfb2 105	Tgfb3 106	Thbs4 107	Tnc 108	Tnf 109	Tnfaip3 110	Fas 111	Vcam1 112
Vegfa 113	Vwf 114	PUC18 115	Blank 116	Blank 117	AS1R2 118	AS1R1 119	AS1 120
Rps27a 121	B2m 122	Hspcb 123	Hspcb 124	Ppia 125	Ppia 126	BAS2C 127	BAS2C 128



Key: **Blue**, control genes; **Red**, genes visibly expressed at this exposure in untreated J774.2 macrophages. See Appendix V for gene abbreviations

5.4.2 Microarray analysis of the effect of LY294002 on the IFN-γ-mediated regulation of macrophage gene expression

J774.2 cells were pretreated with LY294002 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which RNA was extracted. Microarray procedures were carried out as detailed in Section 5.4.1. Relative expression levels were determined using GEArray™ Expression Analysis Software (SuperArray Bioscience Corporation). Statistical analysis of the data from three independent experiments was performed according to the methods described in Section 2.8.7 (see Figure 5.9 for strategy). Table 5.1 lists the genes that were consistently expressed in all three experiments and in the case of genes up- or downregulated in response to IFN-γ, for which this effect was significant. Statistical analysis accurately represents visible data as illustrated in Figure 5.12 for the induction of MCP-1 expression by IFN-γ and the inhibition of this response in the presence of LY294002. For the genes listed in Table 5.1: the relative level of expression; the fold change with IFN-γ treatment; and (for those regulated by IFN-γ) the inhibitory effect (%) of LY294002 on the response, is shown. Relative expression is normalised to the expression of the LPL gene (100%), which gave the highest signal of the genes listed. Of the 66 genes listed the expression of 50 was induced in response to IFN-γ and 3 suppressed, while 6 were unaffected by the cytokine according the the criteria detailed in Section 2.8.7. In the division of IFN-γ-responsive genes according to the action of LY294002, a lower limit of 55% inhibition of the response was set for classification as LY294002-sensitive. Of the 50 genes induced by IFN-γ in the study, the upregulation of 39 genes was inhibited by LY294002 by more than 55%.

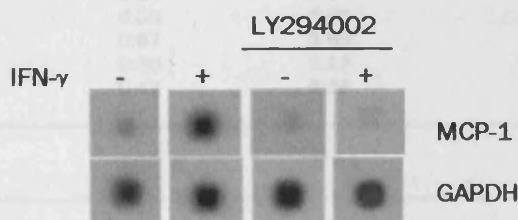


Figure 5.12 Visible representation of the upregulation of MCP-1 gene expression by IFN-γ and the inhibition of this response in the presence of LY294002

J774.2 cells were pretreated with LY294002 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which total RNA was extracted. RNA was converted to biotin-labelled cRNA that was hybridised to the Oligo GEArray™ Mouse Atherosclerosis Microarray and visualised by chemiluminescent detection. Results are representative of three independent experiments

Table 5.1 Effect of LY294002 on IFN-γ-mediated regulation of macrophage gene expression

IFN-γ	LY	GENE	UT	IFN-γ	FOLD CHANGE IFN-γ	% INHIBITION LY
Induced	>55% Inhibition	Msr1	6.28	10.50	1.67	100
		Ccl2 (MCP-1)	6.23	119.43	19.17	96.6
		Il10	4.62	14.49	3.13	79.2
		Ccr2*	4.42	10.73	2.43	98.2
		Bax	2.64	10.02	3.80	100
		Cd36	2.56	8.31	3.24	89.7
		Sell	2.48	5.88	2.37	84.0
		Csf3	2.10	7.15	3.41	100
		Ccl5	1.70	11.96	7.05	67.8
		Ccl20	1.58	6.95	4.38	63.9
		Pdgfb	1.33	11.83	8.87	99.3
		Tnc	1.18	3.98	3.37	62.1
		Itgb2	1.13	4.74	4.21	100
		Fabp3	1.08	3.86	3.57	83.0
		Itgax	1.04	4.22	4.08	96.1
		Ppard	0.99	4.74	4.79	97.1
		Birc3	0.95	10.85	11.39	72.5
		Serpinb2	0.95	3.65	3.85	57.4
		Mmp9	0.94	4.36	4.65	71.6
		Cxcl1	0.77	9.05	11.76	68.8
		Sele	0.76	3.86	5.06	100
		Ctgf	0.75	4.43	5.92	97.5
		Itgb3	0.73	5.94	8.08	94.6
		Ldlr	0.73	4.32	5.90	97.5
		Ifng	0.73	9.92	13.66	90.9
		Lcat	0.72	4.83	6.71	92.4
		Ppara	0.71	4.12	5.82	100
		Soat2	0.68	3.42	5.03	61.6
		Tgfb2	0.57	3.47	6.10	59.5
		Pdgfrb	0.44	3.66	8.31	100
		Il4	0.44	8.25	18.75	66.7
		Lypla1	0.44	3.01	6.88	100
		Il5	0.43	3.68	8.46	100
		Vcam1	0.40	2.93	7.27	85.7
		Ccl11	0.37	2.42	6.50	100
		Cdh5	0.31	2.45	7.86	86.2
		Il1a	0.27	2.66	9.85	93.9
		Il2	0.25	3.71	14.71	68.6
		Apoa2	0.76	2.73	3.58	55.6
	<55% Inhibition	Tnf	4.30	15.40	3.59	45.9
		Icam2	3.39	9.19	2.71	0
		Npy	2.41	6.19	2.57	30.2
		Bcl2a1a	1.67	4.03	2.41	0
		Icam1	1.58	12.95	8.19	0
		Nr1h3	1.41	4.89	3.46	0
		Fga	1.20	4.27	3.57	28.8
		Il13	0.88	9.03	10.30	30.4
		Ccr1	0.67	3.63	5.39	0
		F7	0.55	3.17	5.81	0
		Fgb	0.42	8.26	19.81	43.6
Suppressed	>55%	Lpl	100	60.04	0.60	100
	<55%	Abca1	7.39	5.72	0.77	40.6
		Scarb1	17.97	12.53	0.70	28.8
No Change		Ptgs1	69.12	77.62	1.123	
		Apoa1	42.76	52.57	1.229	
		Nfkb1	40.60	55.09	1.357	
		Cd44	14.66	16.05	1.095	
		Adpf	6.21	7.10	1.143	
		Tgfb3	5.90	6.06	1.027	

5.4.3 Validation of microarray data by RT-PCR analysis

Microarray data indicated an induction of almost 20-fold for MCP-1 expression in response to IFN-γ and only 2.4-fold for CCR2, both of which were attenuated in the presence of LY294002. This is consistent with the RT-PCR data presented previously. In order to further validate the microarray data, RT-PCR analysis was carried out for selected genes from each category to confirm the responses to IFN-γ and LY294002. J774.2 cells were pretreated with LY294002 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which RNA was extracted. RT-PCR analysis was performed using primers specific for: MCP-1; CD36; RANTES; MMP9; IL-10; TNF-α; ICAM-1; LPL; ABCA1; SR-B1; NF-κB; CD44; and GAPDH, as the control gene to which microarray data was normalised. PCR products were resolved by agarose gel electrophoresis and bands of the appropriate size were obtained.

For the majority of these genes the RT-PCR data was consistent with the microarray results shown in Table 5.1. However discrepancies were found concerning the genes IL-10 and LPL. RT-PCR analysis showed a marked suppression in the expression of IL-10 in response to IFN-γ, in contrast to the induction reported in the microarray data. For LPL gene expression, the suppressive effect of IFN-γ was consistent between the RT-PCR and microarray data as well as previous studies in our laboratory. Incubation with LY294002 during 24h stimulation with IFN-γ, has been found to prevent the inhibition in expression mediated by the cytokine in THP-1-derived macrophages (Evans, S., personal communication) consistent with the microarray data presented in Table 5.1. RT-PCR analysis in J774.2 cells however, with 3h IFN-γ treatment, does not show such a marked inhibition with LY294002 although densitometric analysis (data not shown) does indicate a small inhibition in the response due to a reduction in the basal level of LPL expression in cells treated with LY294002. RT-PCR data is summarised in Figure 5.13 and is representative of three independent experiments.

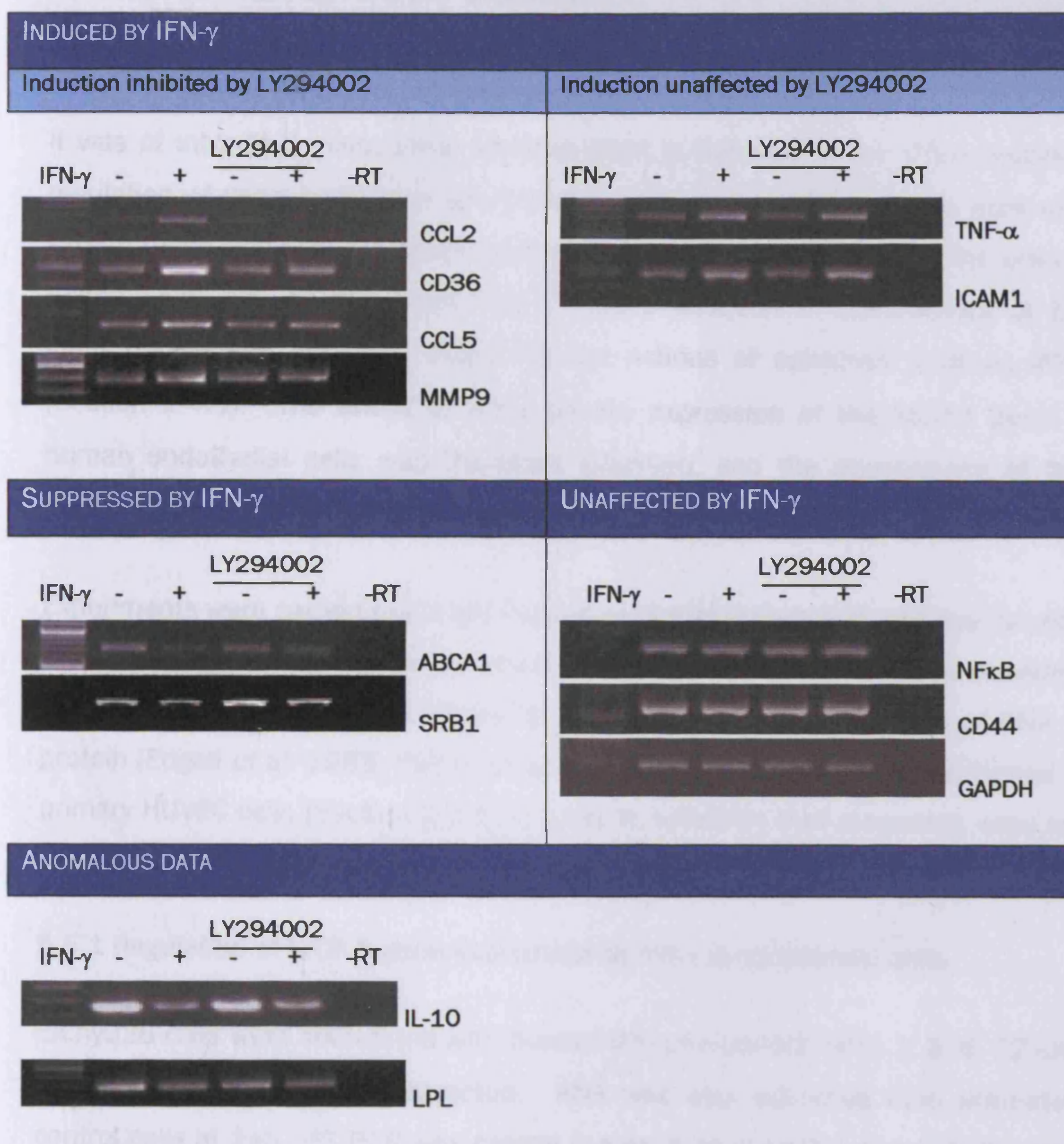


Figure 5.13 Validation of microarray data by RT-PCR analysis

J774.2 cells were pretreated with LY294002 (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h after which total RNA was extracted. RT-PCR analysis was carried out using primers specific for: MCP-1; CD36; RANTES; MMP9; IL-10; TNF-α; ICAM-1; LPL; ABCA1; SR-B1; NF-κB; CD44; and GAPDH and the products resolved by agarose gel electrophoresis. –RT denotes a reaction for which no reverse transcriptase was included for cDNA synthesis (using vehicle-treated RNA). Results are representative of at least three independent experiments.

5.5 Role of PI3K-Dependent Signalling in the Regulation of MCP-1 Gene Expression by IFN-γ in Endothelial Cells

It was of interest to determine whether PI3K is involved in the IFN-γ-mediated regulation of gene expression specifically in macrophages or if future work may extend these findings into other cells of the atherosclerotic plaque. The arterial endothelium has an important role in the production of chemokines at the inflammatory site and is a target for the actions of cytokines such as IFN-γ (Section 1.4.3). The effect of IFN-γ on the expression of the MCP-1 gene in human endothelial cells was therefore analysed, and the dependence of this response on PI3K-mediated signalling.

Experiments were carried out in the human endothelial EA.hy926 cell line (Section 2.3.1). Cells of the EA.hy926 line closely represent functional ECs while providing a homogeneous system that allows for the extraction of a good yield of RNA or protein (Edgell *et al.* 1983, Rieber *et al.* 1993). Key data were also confirmed in primary HUVEC cells (Section 2.3.5) in order to establish that responses were not peculiar to the transformed cells.

5.5.1 Regulation of MCP-1 gene expression by IFN-γ in endothelial cells

EA.hy926 cells were stimulated with human IFN-γ for periods of 0, 1, 3, 6, 12 and 24h after which RNA was extracted. RNA was also extracted from untreated control cells at 24h. RT-PCR was carried out for human MCP-1 and GAPDH using the primers and conditions detailed in Table 2.5. Figure 5.14 summarises the change in mRNA levels for MCP-1 in response to IFN-γ, over the duration of the time course. Figure 5.14A illustrates the PCR products fractionated by agarose gel electrophoresis. The band densities were normalised to the expression of GAPDH and the fold induction in comparison to the mRNA expression at 0h (assigned as 1) was plotted in relation to time (h) (Figure 5.14B). While maximal MCP-1 mRNA levels were observed following 3h stimulation with IFN-γ as in macrophages, levels declined at 12h and 24h indicating the existence of a refractory mechanism in these cells that is not present in macrophages.

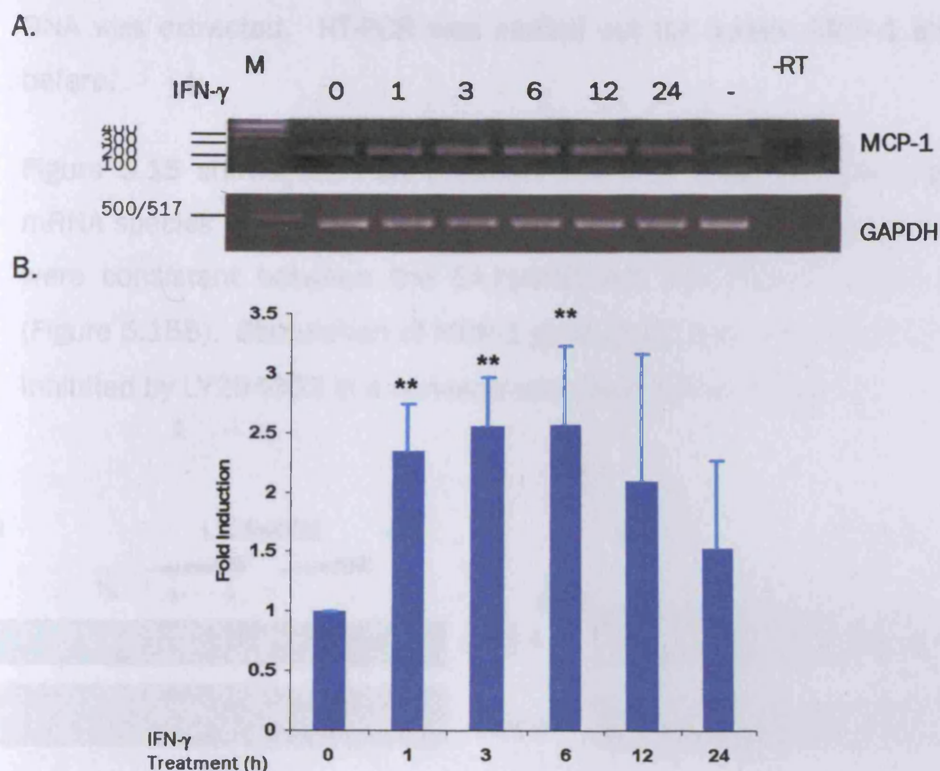


Figure 5.14 Regulation of MCP-1 gene expression by IFN-γ in endothelial cells

EA.hy926 endothelial cells were exposed to human IFN-γ for the indicated time. Total RNA was isolated and subjected to RT-PCR using primers against human MCP-1 and GAPDH. The amplification products were analysed by agarose gel electrophoresis (A). The position of the appropriate product and the size of the DNA markers are indicated. Panel B shows the fold induction (mean ±SD) in the expression of MCP-1 normalised to the expression of GAPDH as determined by densitometric analysis from three independent experimental series. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using untreated RNA). **P<0.01

5.5.2 Effect of LY294002 on the IFN-γ-mediated induction of MCP-1 gene expression

The effect of LY294002 on the IFN-γ-mediated induction of MCP-1 gene expression was studied in both the EA.hy926 cell line and in HUVECs to ensure that responses are not a peculiar property of the EA.hy926 cell line. Cells were pretreated with LY294002 (5μM and 20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h after which

RNA was extracted. RT-PCR was carried out for human MCP-1 and GAPDH as before.

Figure 5.15 shows the PCR products obtained using primers specific to each mRNA species and the band intensities in relation to GAPDH expression. Results were consistent between the EA.hy926 cell line (Figure 5.15A) and HUVECs (Figure 5.15B). Stimulation of MCP-1 gene expression following IFN-γ treatment is inhibited by LY294002 in a concentration dependent manner.

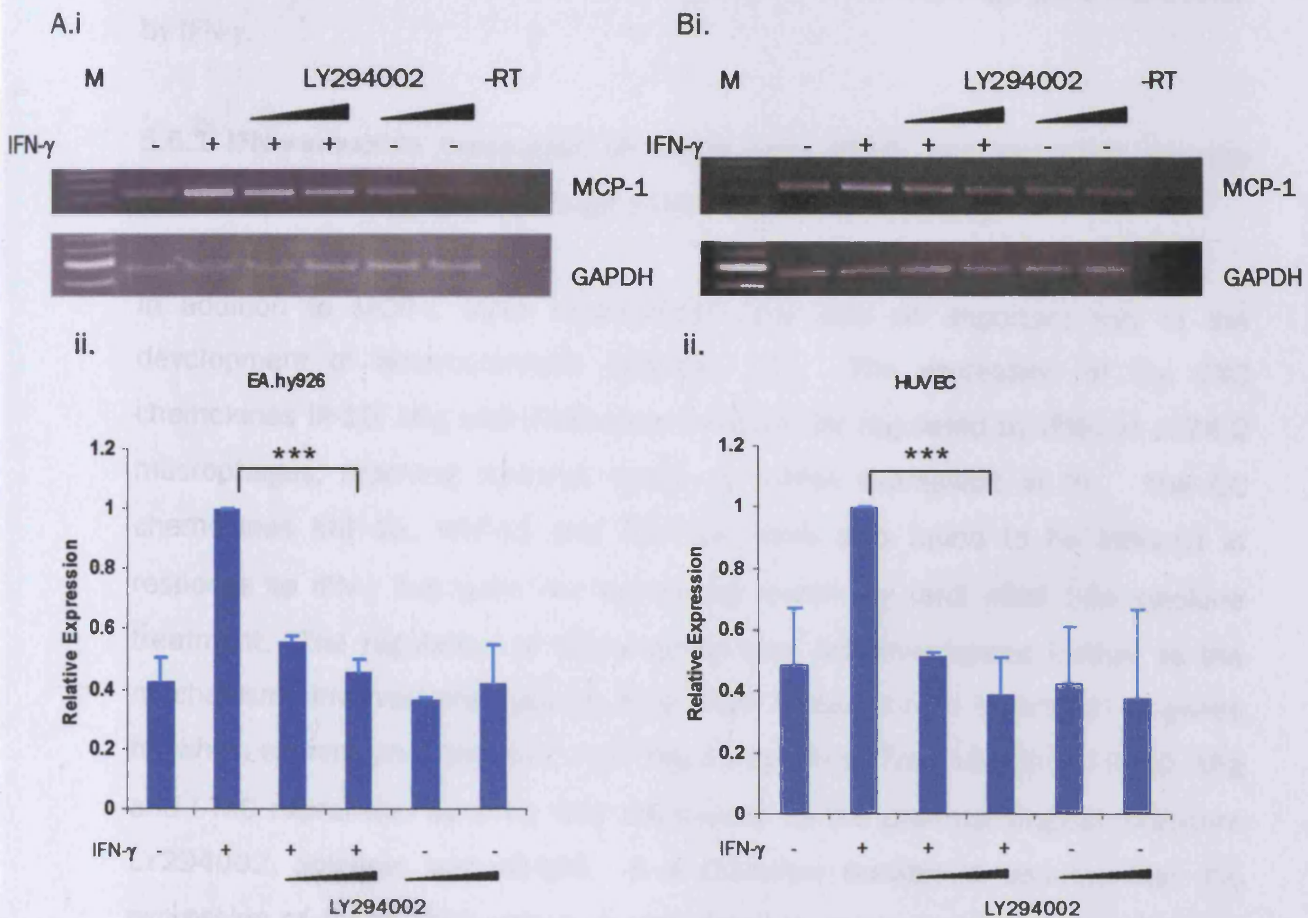


Figure 5.15 Effect of LY294002 on the IFN-γ-mediated induction of MCP-1 gene expression in endothelial cells

EA.hy926 (A) and HUVECs (B) were pretreated with LY294002 (5μM and 20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h after which total RNA was extracted. RT-PCR analysis was carried out using primers specific for human MCP-1 and GAPDH and the products resolved by agarose gel electrophoresis. –RT denotes a reaction for which no reverse transcriptase was included for cDNA synthesis (using vehicle-treated RNA). (A.i and B.i). Densitometric analysis was carried out and presented as the relative expression (mean ±SD) normalised to the expression of GAPDH (A.ii and B.ii). Results are representative of three independent experiments in EA.hy926 cells and two in HUVECs. ***P<0.001.

5.6 DISCUSSION

With the aim of expanding the findings regarding the role of PI3K in IFN-γ-mediated regulation of gene expression, further studies were carried out into the regulation of subsets of genes by IFN-γ in macrophages. Initial investigations involved RT-PCR analysis of the regulation of the expression of chemokines and the receptor CCR2 by IFN-γ. Gene array technology was then utilised to show a selective requirement for PI3K in the regulation of macrophage gene expression by IFN-γ.

5.6.1 IFN-γ-inducible expression of chemokines IP-10, Mig and I-TAC and the receptor CCR2 is regulated through a PI3K-dependent pathway

In addition to MCP-1 other chemokines also play an important role in the development of atherosclerosis (Section 1.5). The expression of the CXC chemokines IP-10, Mig and I-TAC were found to be regulated by IFN-γ in J774.2 macrophages, reaching maximal levels of mRNA expression at 3h. The CC chemokines MIP-1α, MIP-1β and RANTES were also found to be induced in response to IFN-γ but were not expressed maximally until after 24h cytokine treatment. The regulation of these genes was not investigated further as the mechanisms involved are likely to differ from those studied in relation to genes for which expression is induced more rapidly by IFN-γ. The induction of IP-10, Mig and I-TAC expression by IFN-γ was attenuated by the pharmacological inhibitors LY294002, apigenin and AG490. It is therefore feasible to assume that the expression of these chemokines is stimulated in response to IFN-γ through the PI3K-dependent pathway responsible for the upregulation of MCP-1 gene expression.

The expression of the MCP-1 receptor, CCR2, was also found to be induced by IFN-γ treatment in J774.2 macrophages. Pretreatment with the transcriptional ihibitor actinomycin D showed that this effect is due to increased transcription of the MCP-1 gene as opposed to increased mRNA stability. Previous studies have presented conflicting data with regards to the regulation of CCR2 by IFN-γ in

monocytes. While Schwarz *et al.* (2002) have demonstrated an induction in CCR2 expression by the cytokine in human mesangial cells, studies in monocytes have shown that CCR2 is downregulated in response to IFN-γ (Tangirala *et al.* 1997, Penton-Roi *et al.* 1998). The authors suggest that this enables the retention of monocytes recruited to an inflammatory site. Similarly the proinflammatory stimulus oxLDL has been found to mediate a decrease in CCR2 expression, in contrast to native LDL which upregulates expression of the receptor (Han *et al.* 1998, Han *et al.* 2000). However following monocytic-differentiation, mature macrophages will not migrate from the inflammatory site. In this situation IFN-γ may induce the expression of CCR2 in order to enhance other cellular responses to the chemokines present. The induction of CCR2 expression by IFN-γ was attenuated by the pharmacological inhibitors LY294002, apigenin and AG490, suggesting that a pathway dependent on JAK2, CK2 and PI3K may be involved in both the regulation of chemokines and certain receptors in response to IFN-γ.

A brief analysis of the promoter region approximately 400bp upstream of the transcriptional start site for the murine IP-10, Mig, I-TAC and CCR2 genes, revealed the presence of putative GAS elements for each of these genes (Appendix VI). Functional data has been obtained for the activation of the murine IP-10 and Mig promoters by IFN-γ (Ohmori and Hamilton 1992, Wright and Farber 1991). These genes are therefore all potentially regulated through STAT1 without the need for *de novo* protein synthesis. There are some variations in the expression profile in response to IFN-γ between these genes, both in the degree of induction and the change in mRNA levels over time. This may be due to the initial conformation of the promoter or the necessity for different combinations of factors regulated through the same, or divergent pathways. Further promoter and biochemical studies are required to determine the precise mechanisms involved.

5.6.2 Microarray analysis reveals a selective role for PI3K in the regulation of IFN-γ-inducible gene expression in macrophages

This study was extended to the global regulation of macrophage gene expression through the use of microarray analysis. These experiments confirmed a selective role for PI3K in IFN-γ-inducible gene expression. The commercial array (SuperArray Bioscience Oligo GEArray™ Mouse Atherosclerosis Microarray) chosen for use in this analysis contained a specifc selection of oligonucleotides representing genes for which a role has been demonstrated in atherosclerosis. As presented in Table 5.1, the expression of 50 of these genes was induced in response to IFN-γ and 3 suppressed, while 6 were unaffected by the cytokine. The high proportion of genes that were found to be induced by IFN-γ may be due to the selection of genes represented on the array. The cytokine is known to have an important role in the pathology of atherosclerosis and this is apparent in the high number of genes regulated by IFN-γ in this study. A number of these genes have been discussed previously in relation to the role of IFN-γ in the progression of atherosclerosis. For instance VCAM-1 and ICAM-1, the IFN-γ-mediated induction of which is particularly notable on the surface of ECs (Cybulsky *et al.* 1993, Chung *et al.* 2002) were shown to also be induced by the cytokine in macrophages (although the level of VCAM-1 expression is relatively low). LPL (Hughes *et al.* 2002) and ABCA1 (Panousis and Zuckerman 2000a), for which expression is suppressed, are also discussed in Section 1.4.6.

The expression of a number of cytokines was found to be induced including IL-4, IL-5, IL-1a, IL-2, TNF-α, IL-13 and IL-10 (although this was not confirmed by PCR validation, see below) and several chemokines and receptors including CCL2 (MCP-1), CCL5 (RANTES) (see below), CCL20 (MIP-3α), CXCL1 (GRO-α), CCL11 (eotaxin), CCR2 and CCR1. Interestingly, the expression of lipoprotein receptors, including Msr1 (SR-A), CD36, LDL-R and Scarb1 (SR-B1), are shown in this study to be regulated by IFN-γ in a contrasting manner to that described previously (Section 1.4.6). IFN-γ is usually considered to act in an atheroprotective manner in the downregulation of the scavenger receptors SR-A and CD36 (Geng and Hansson 1992, Nakagawa *et al.* 1998). However this response is observed

following prolonged stimulation with IFN-γ for 24-36h and the effect of shorter term stimulation may be different. A study by Grewal *et al.* (2001) have proposed that scavenger receptor expression is differentially regulated over monocytic differentiation and also in response to long and short term treatment with IFN-γ. They have shown an induction in SR-A expression in THP-1 cells cultured in the presence of PMA for up to 24h following a further 24h stimulation with IFN-γ. This is mediated through binding to a GAS element in the promoter. Longer periods of treatment with IFN-γ resulted in reduced levels of SR-A expression (Grewal *et al.* 2001). The results of microarray analysis indicated that IFN-γ acts in a similarly atherogenic manner by suppressing the expression of the atheroprotective HDL-receptor SR-B1 (Huszar *et al.* 2000).

In the division of IFN-γ-responsive genes according to the action of LY294002, an inhibition of the response of less than 55% was classed as unaffected. Of the 50 genes induced by IFN-γ in the study the upregulation of 39 of these was inhibited by LY294002 by more than 55%. Although the action of LY294002 in the inhibition of the IFN-γ response is clearly selective, the majority of genes in this array are regulated by the cytokine in a PI3K-dependent manner. Again it is uncertain whether this is an accurate reflection of the proportion of genes regulated through this pathway or if the selection of genes involved in atherosclerotic changes has distorted these findings. If this were the case PI3K may preferentially function in the regulation of proinflammatory, proatherogenic mediators in response to IFN-γ and as such would provide a potential therapeutic target for the treatment of atherosclerosis. For instance the IFN-γ-inducible expression of the predominantly antiinflammatory cytokine IL-13 is not affected by LY294002 treatment, while that of the proinflammatory cytokines IL-5, IL-1α, IL-2 and IL-4 (which has been shown to act in both a pro- and antiatherogenic manner) is inhibited in the presence of LY294002 (von der Thusen *et al.* 2003). This is not the case for all the genes for which the IFN-γ response is unaffected by LY294002 treatment. For example the cell adhesion molecules ICAM-1 and VCAM-1 are both considered to have an equal impact on the development of atherosclerosis (although the function of these molecules is less well characterised in macrophages than in ECs) but the IFN-γ-mediated induction is

differentially responsive to LY294002. Further studies are clearly required before firm conclusions can be drawn in relation to the role of PI3K in the regulation of pro- and antiatherogenic genes in response to IFN-γ.

Data from microarray experiments was consistent with previous findings from RT-PCR analysis, showing an induction of almost 20-fold for MCP-1 expression in response to IFN-γ and only 2.4-fold for CCR2, both of which were attenuated in the presence of LY294002. However in order to further validate the microarray data, RT-PCR analysis was carried out for selected genes from each category to confirm the responses to IFN-γ and LY294002. As shown in Figure 5.13, for the majority of these genes the RT-PCR data was consistent with the microarray results. The expression of RANTES was also found to be upregulated in response to only 3h stimulation with IFN-γ in this experiment, an effect seen only marginally in the results presented in Figure 5.2. RT-PCR confirmed this response and the inhibitory effect of LY294002 at this time point. The discrepancy here may be due to the limitations of semi-quantitative RT-PCR in the analysis of genes that are not dramatically induced by the relevant stimulus.

As detailed in Section 5.4.3 anomalous data was presented with respect to the genes IL-10 and LPL. A literature review supports the finding that IFN-γ inhibits the expression of IL-10 as shown in RT-PCR data (Li *et al.* 1997, Chromarat *et al.* 1993). Interestingly, IL-10 expression has been shown to be regulated by the transcription factors Sp1 and Sp3 and IFN-γ may mediate the suppression of gene expression through a mechanism similar to that involved in the regulation of the LPL gene (Hughes *et al.* 2002, Tone *et al.* 2000). The possibility must therefore be considered, that a second transcript that is upregulated by IFN-γ also has the ability to bind to the oliogonucleotide sequence representing IL-10 on the array. For LPL gene expression, while the suppressive effect of IFN-γ was consistent between the RT-PCR and microarray data, as well as previous studies in our laboratory (Hughes *et al.* 2002), a difference was observed in the level of inhibition of the IFN-γ response following incubation with LY294002. It is uncertain from this data whether PI3K is involved in the suppression of LPL following 3h stimulation with IFN-γ. However, LY294002 has been found to inhibit

this response during 24h stimulation with the cytokine, the time point at which LPL is maximally suppressed by IFN-γ in J774.2 cells. The results may differ here due to the difficulties of exact quantification of expression in either RT-PCR or microarray analysis.

These findings highlight the need for caution in the interpretation of microarray data and for independent confirmation by RT-PCR experiments. This does not mean that such experiments do not provide important information regarding the global role of mediators in signal transduction as well as indicating genes for which further mechanistic studies would be of interest. Further studies that may arise from the microarray data presented here include an analysis of the differential pathways regulating VCAM-1 and ICAM-1 expression and an extension of the investigation into the regulation of chemokine and chemokine receptor expression by IFN-γ. Of particular interest may be the PI3K-independent induction of CCR1 by the cytokine in comparison to the induction of CCR2. Dominant negative or siRNA based experiments should also be carried out to confirm the role of PI3K in the regulation of genes of particular interest. Dependence of the regulation of these genes on the IFN-γ-mediated activation of JAKs, STAT1 and CK2 should also be investigated by dominant negative or siRNA-based approaches.

5.6.3 A PI3K-dependent pathway regulates the IFN-γ-mediated induction of MCP-1 expression in endothelial cells

The arterial endothelium has an important role in the production of chemokines at the inflammatory site, and in atherosclerosis, and is a target for the actions of cytokines such as IFN-γ (Section 1.4.3). In order to determine whether PI3K is involved in the IFN-γ-mediated regulation of gene expression specifically in macrophages, or if future work may extend these findings into other cells of the atherosclerotic plaque, the effect of IFN-γ on the expression of the MCP-1 gene in human ECs was analysed. For initial studies in these cells the human endothelial EA.hy926 cell line was utilised. As in macrophages maximal MCP-1 mRNA expression in these cells was observed following 3h stimulation with IFN-γ.

However mRNA levels declined at 12h and 24h indicating the existence of a refractory mechanism in these cells that is not present in J774.2 macrophages. This effect has been observed previously by Zhou *et al.* (2001) in human astrocytoma cells.

The dependence of this response on PI3K-mediated signalling was assessed by pretreatment of ECs with LY294002. This experiment was performed in both EA.hy926 cells and primary HUVECs. IFN-γ induced the expression of MCP-1 following 3h treatment in both cell types indicating that this was not a peculiar property of the EA.hy926 cell line. LY294002 was also shown to inhibit the IFN-γ-mediated induction of MCP-1 expression in EA.hy926 cells and HUVECs in a dose dependent manner.

CHAPTER SIX:

MECHANISMS IN THE REGULATION OF ICER GENE EXPRESSION BY IFN-γ

CHAPTER 6. MECHANISMS IN THE REGULATION OF ICER GENE EXPRESSION BY IFN-γ

6.1 INTRODUCTION

As discussed in Section 3.2-3.3, IFN-γ was found to stimulate a dramatic increase in ICER expression in J774.2 macrophages, maximal levels resulting from between 2h and 6h of stimulation. The IFN-γ-mediated induction of ICER was found to be attenuated by the CK2 inhibitor apigenin. The phosphorylation of CREB at Ser-133 (known to induce the *trans*-activation potential of this protein) was also shown to be increased by IFN-γ treatment and this response was inhibited by apigenin. Although CK2 is considered to be a constitutively active kinase, the activity of the α-subunit was demonstrated to be increased by treatment with IFN-γ, maximal activation being achieved after 2h stimulation (Mead *et al.* 2003).

6.1.1 Role of CK2 and the JAK-STAT pathway in the regulation of ICER expression by IFN-γ

Although the use of apigenin indicated that CK2 may be involved in the regulation of MCP-1 and SOCS-1 expression in response to IFN-γ, as well as ICER (Figure 3.3), the induction of ICER expression was not found to be dependent on the activation of JAK2 as for MCP-1 and SOCS-1 (Figure 4.2). Nor was there a requrement for PI3K (Figure 3.5). This raises the possibility that ICER gene expression is regulated in response to IFN-γ in a novel JAK-STAT-independent pathway. This chapter focuses on further studies carried out into the mechanisms involved in the IFN-γ-stimulated upregulation of ICER expression with the aim of establishing the function of CK2, JAK-STAT signalling and other potential mediators in this response.

6.1.2 Role of other mediators in the regulation of ICER expression by IFN-γ

Previous work in our laboratory, regarding the expression of ICER in response to IFN-γ, has utilised a number of inhibitors. These include: H89, an inhibitor of PKA; bisindoylmaleimide, a PKC inhibitor; SB202190, a p38 MAPK inhibitor; PD98059, an ERK inhibitor; W7, a calcium-calmodulin-dependent protein kinase inhibitor; and the JAK2 inhibitor, AG490. None of these compounds were found to affect signalling by IFN-γ with respect to the expression of ICER (Mead *et al.* 2003). The c-Jun NH_2-terminal kinase/stress activated protein kinase (JNK/SAPK) pathway has not been studied for a potential role in the regulation of ICER.

JNK is a member of the MAPK family of signal transducing proteins and is activated through MAPK signalling cascades. JNK/SAPK is activated by environmental stress, cytokines and other stimuli and regulates the activity of a variety of transcription factors including AP-1, Sp1, NF-κB as well as ATF-2 and CREB. The pathway is required for the regulation of the inflammatory response, proliferation and apoptosis (Manning and Davis 2003, Wang et al 2000a, Sanchez *et al.* 1994). Several recent studies have linked the actions of CK2 to the activation of a JNK signalling cascade (Hilgard *et al.* 2004, Riga *et al.* 2004, Fleming *et al.* 2000).

6.1.3 Experimental strategy

The initial screen using a panel of pharmacological agents only revealed a role for CK2 in the IFN-γ-mediated regulation of ICER gene expression. With the aim of identifying a further potential effector of the IFN-γ response, a pharmacological inhibitor of JNK activity was used to establish whether this kinase is involved in the regulation of ICER expression by the cytokine. In order to confirm the role of CK2 in the induction of ICER expression by IFN-γ a dominant negative mutant of the kinase was used in conjunction with RT-PCR analysis and the transfection of a luciferase reporter construct modelling the ICER promoter. Dominant negative mutants for JAK1, JAK2 and STAT1 were also employed to establish whether there is a requirement for the JAK-STAT pathway in this response (see Figure 6.1 for the overall experimental strategy).

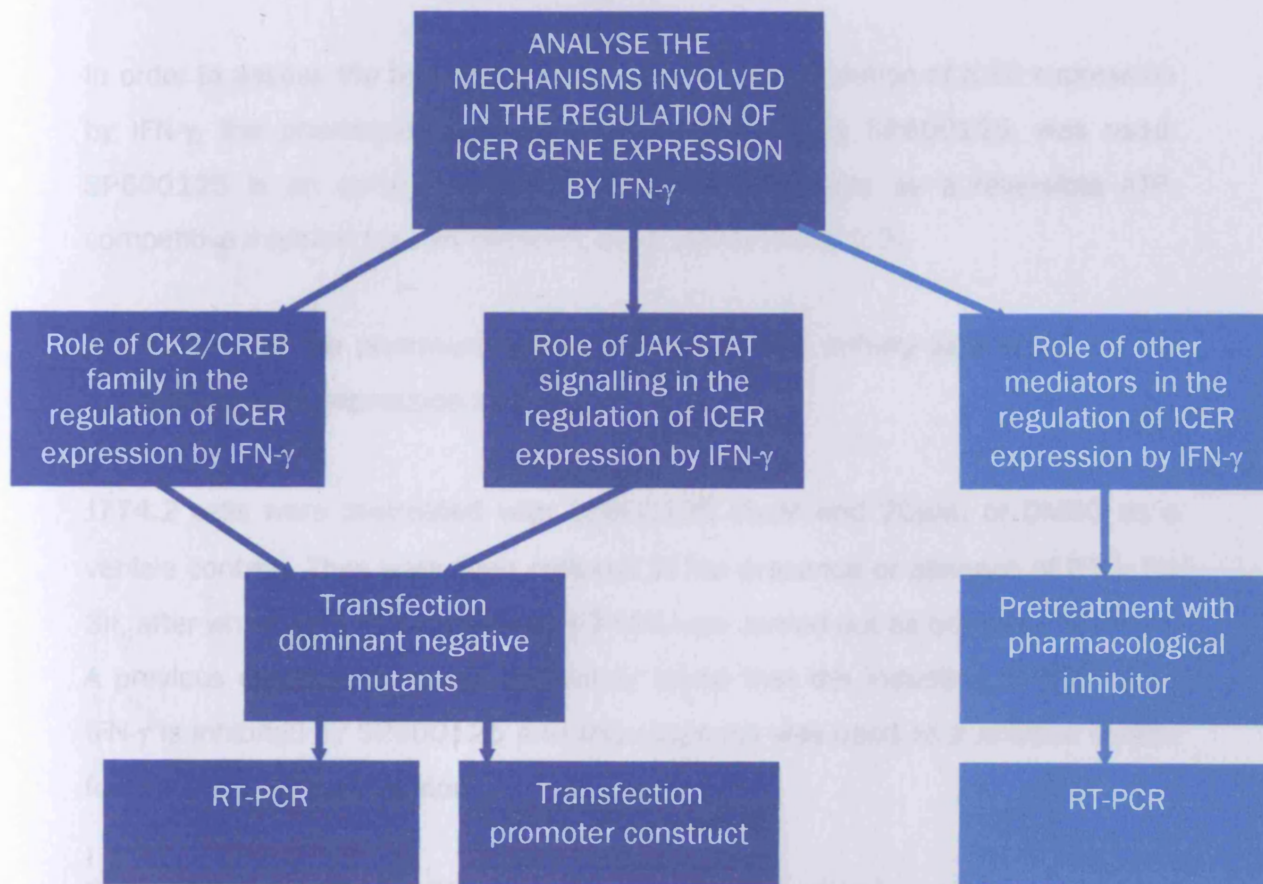


Figure 6.1 Experimental strategy

Pharmacological inhibitors were used with the aim of identifying further potential effectors in the IFN-γ-mediated regulation of ICER expression. In order to confirm the role of CK2 in the induction of ICER expression by IFN-γ a dominant negative mutant of the kinase was used in conjunction with RT-PCR analysis and the transfection of a luciferase reporter construct modelling the ICER promoter. Dominant negative mutants for JAK1, JAK2 and STAT1 were also employed to establish whether there is a requirement for the JAK-STAT pathway in this response

6.2 Effect of Pharmacological Inhibitors on IFN-γ-Stimulated Expression of ICER

In order to assess the role of the kinase JNK in the regulation of ICER expression by IFN-γ, the pharmacological inhibitor of JNK activity SP600125, was used. SP600125 is an anthrapyrazolone compound that acts as a reversible ATP-competitive inhibitor for JNK (Bennett *et al.* 2001) (Table 2.2).

6.2.1 Effect of the pharmacological inhibitor of JNK activity SP600125 on the induction of ICER expression by IFN-γ

J774.2 cells were pretreated with SP600125 (5μM and 20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h, after which RNA was extracted. RT-PCR was carried out as before for ICER I/II. A previous observation in our laboratory found that the induction of SOCS-1 by IFN-γ is inhibited by SP600125 and this response was used as a positive control for the action of the inhibitor.

Figure 6.2 shows the PCR products resolved by agarose gel electrophoresis (Figure 6.2A) and band densities normalised to β-actin (Figure 6.2B). The level of ICER I/II mRNA did not differ between IFN-γ-stimulated samples and those treated with SP600125 in addition to IFN-γ. In contrast the induction of SOCS-1 by IFN-γ was inhibited by SP600125 in a concentration dependent manner. This is an interesting observation and suggests that JNK may also have a role in JAK-STAT signalling. However, due to time constraints, this was not persued in the current study.

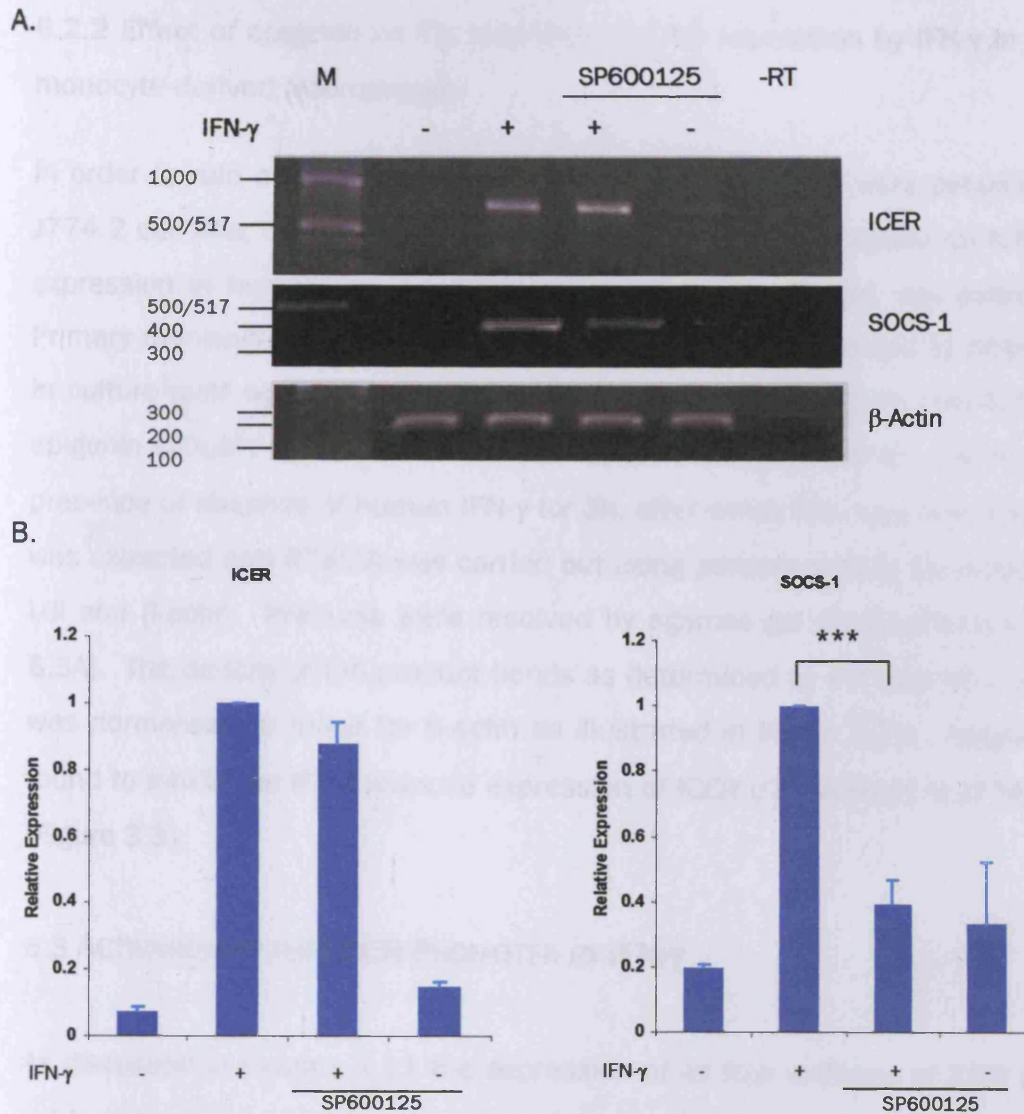


Figure 6.2 Effect of SP600125 on the induction of ICER expression by IFN-γ

J774.2 cells were pretreated with SP600125 (5μM and 20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against ICER, SOCS-1 and β-actin as indicated. The amplification products were analysed by agarose gel electrophoresis (A). The size of the DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis from three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). ***P<0.001.

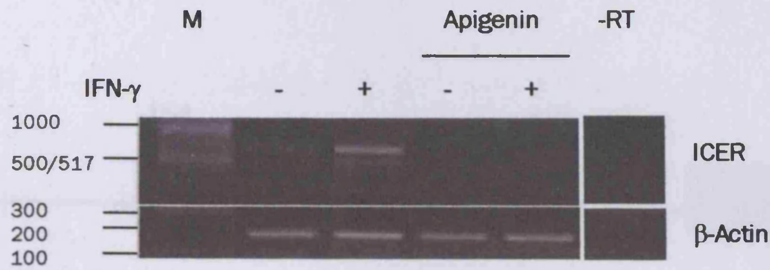
6.2.2 Effect of apigenin on the induction of ICER expression by IFN-γ in primary monocyte-derived macrophages

In order to rule out the possibility that the results obtained were peculiar to the J774.2 cell line, the effect of IFN-γ and the CK2 inhibitor apigenin on ICER gene expression in human primary monocyte-derived macrophages was investigated. Primary monocytes were isolated from human blood and allowed to differentiate in culture over seven days (Section 2.3.4). Macrophages were pretreated with apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h, after which RNA was extracted. RNA was extracted and RT-PCR was carried out using primers specific for human ICER I/II and β-actin. Products were resolved by agarose gel electrophoresis (Figure 6.3A). The density of the product bands as determined by densitometric analysis was normalised to those for β-actin as illustrated in Figure 6.3B. Apigenin was found to inhibit the IFN-γ-induced expression of ICER I/II mRNA as in J774.2 cells (Figure 3.3).

6.3 ACTIVATION OF THE ICER PROMOTER BY IFN-γ

As discussed in Section 1.11 the expression of all four isoforms of ICER (I, Iγ, II, IIγ) is controlled by an internal, alternative promoter (P_2) in the CREM gene. This promoter region contains four tandem CRE-related sequences known as cAMP autoregulatory elements (CAREs) as depicted in Figure 6.4.

A.



B.

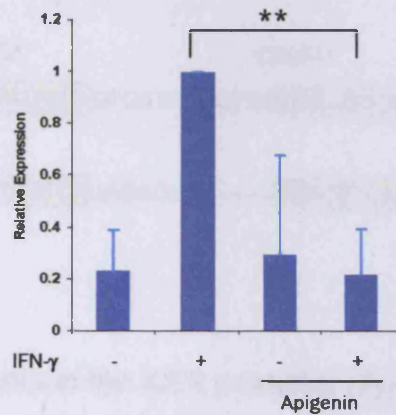


Figure 6.3 Effect of apigenin on the induction of ICER expression by IFN-γ in primary monocyte-derived macrophages

Primary monocyte-derived macrophages were pretreated with apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against hICER and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of DNA markers are indicated. Panel B shows the relative expession (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis from two independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle treated RNA). **P<0.01.

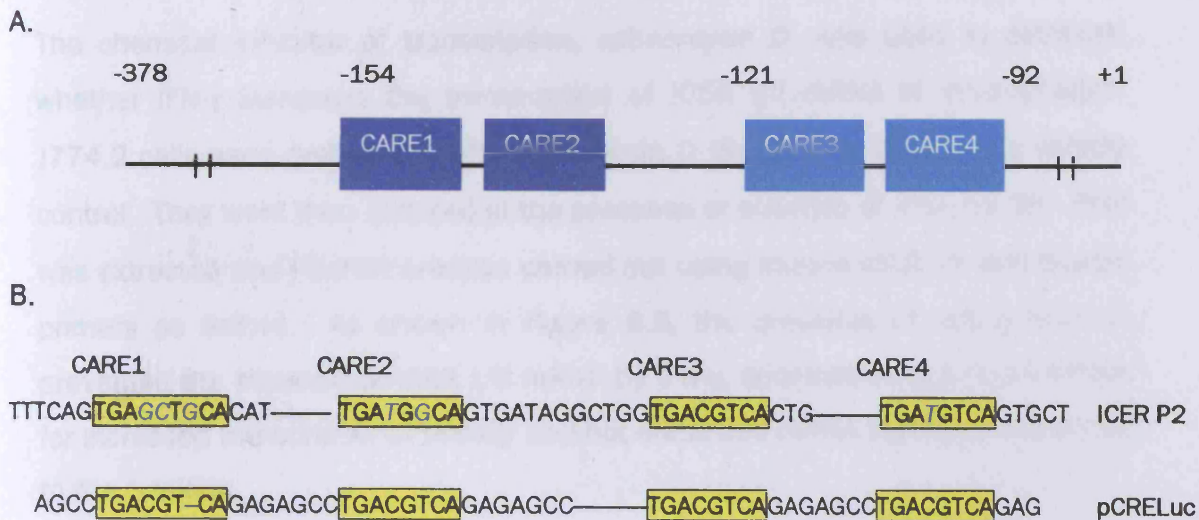


Figure 6.5 Functional elements in the ICER promoter (P_2)

Schematic representation of the alternative promoter (P_2) of the CREM gene that regulates the expression of the ICER transcript, showing the position of four tandem CRE-like elements known as CAREs (A). CARE3 and CARE4 conform to the consensus for classical CREs (Krueger *et al.* 1999). The luciferase reporter construct, pCRELuc (Stratagene) contains four tandem CRE consensus sites and was used to model the ICER promoter in transfection experiments (Section 6.3). Panel B illustrates a comparison of the sequence of the regulatory region of pCRELuc and the CAREs of the ICER promoter.

6.3.1 IFN-γ-mediated upregulation of ICER I/II mRNA levels in macrophages is dependent on transcriptional activity but not mRNA stability

The chemical inhibitor of transcription, actinomycin D, was used to establish whether IFN-γ increases the transcription of ICER I/II mRNA in macrophages. J774.2 cells were pretreated with actinomycin D (5μg/ml) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. RNA was extracted and RT-PCR analysis carried out using murine ICER I/II and β-actin primers as before. As shown in Figure 6.5, the presence of actinomycin D prevented the increase of ICER I/II mRNA by IFN-γ, demonstrating a requirement for increased transcriptional activity and not enhanced mRNA stability in response to the cytokine.

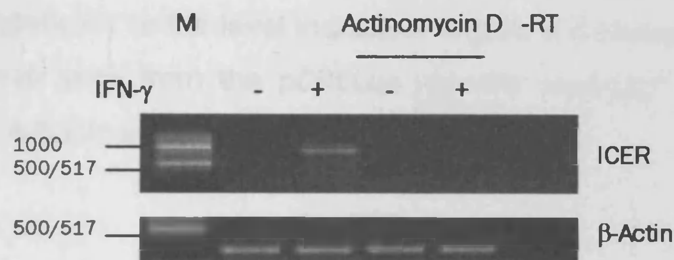


Figure 6.5 Effect of actinomycin D on the induction of ICER expression by IFN-γ

J774.2 macrophages were pretreated with actinomycin D (5μg/ml) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against ICER I/II and β-actin. The amplification products were analysed by agarose gel electrophoresis. The size of the DNA markers are indicated. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). Results shown are representative of two independent experiments.

6.3.2 Expression from a reporter construct containing tandem CRE consensus sites is inducible by IFN-γ

A luciferase reporter construct (pCRELuc) in which expression of the luciferase gene is controlled by a TATA box linked to four CRE consensus enhancer elements, as described in Section 2.4, was obtained from Stratagene and used to model the response of the P_2 promoter regulating ICER expression. A comparison

of the insert sequence containing the CREs to the sequence of the CAREs of the ICER promoter is given in Figure 6.4B. In order to investigate whether tandem CREs similar to those in the ICER promoter can support IFN-γ-inducibility, pCRELuc was transfected into U937 cells, differentiated in the presence of PMA, and the regulatory effect of IFN-γ treatment measured.

The pCRELuc plasmid was transfected into U937 cells by the SuperFect™ (Qiagen) transfection method and treated with IFN-γ for 12h as described previously (Section 3.5). Tranfected cells were harvested and extracts prepared for assay of reporter gene activity. All transfections were carried out with samples in triplicate for at least three independent experiments. Results are presented as the mean fold induction (±SD) (basal level assigned as 1). A standard statistical t-test (Appendix IV) was performed on the data and changes in expression were found to be significant to the level indicated. Figure 6.6 shows that transcription of the luciferase gene from the pCRELuc reporter construct was increased by approximately 4-5 fold in cells treated with IFN-γ.

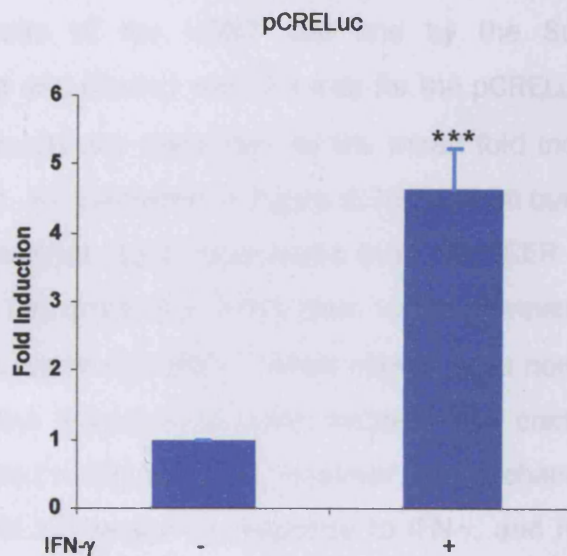


Figure 6.6 Activation of a promoter regulated by tandem CREs by IFN-γ in macrophages

U937 cells were transfected with the reporter construct pCRELuc and treated with IFN-γ for 12h. Relative counts were normalised to protein concentration and presented as the mean fold induction (±SD) in response to IFN-γ, in relation to basal expression. Results are representative of five independent experiments. ***P<0.001.

6.3.3 Generation of a reporter construct containing tandem CAREs

A reporter construct based on the pGL2-Promoter plasmid backbone and regulated by a 63bp region of the ICER promoter, containing four CAREs, was generated to determine if the regulation by IFN-γ corresponds to that of pCRELuc. Sense and antisense oligonucleotides were synthesised (Sigma-Genosys, UK) and annealed (Section 2.7.2.1) to form double-stranded DNA representing the sequence of the four CAREs of the ICER promoter (P_2) and containing XhoI and XmaI restriction sites at the 5' and 3' ends respectively (Appendix III). This sequence was cloned into the pGL2-Promoter luciferase reporter plasmid by the method described in Section 2.5.12 and the sequence of the insert was verified by sequencing (Lark, UK) (Section 2.5.12.5). Figure 6.7A shows the PCR product resulting from a reaction with the primers GL1 and GL2 (Table 2.5) of the ICER4xCARE plasmid, confirming the presence of an insert of the correct size (PCR product for empty vector, 135bp; plus insert, 172bp).

The ICER4xCARE construct, and the empty vector pGL2-Promoter, were transfected into cells of the U937 cell line by the SuperFect™ (Qiagen) transfection method and treated with IFN-γ as for the pCRELuc reporter construct (Section 6.3.2). Results are presented as the mean fold induction (±SD) (basal level assigned as 1). As illustrated in Figure 6.7B, a small but significant increase was observed in reporter gene expression from the ICER 4xCARE plasmid in response to IFN-γ. The empty pGL2-Promoter vector however, displayed reduced activation upon treatment with IFN-γ. When counts were normalised to those for the empty vector the induction of ICER 4xCARE was comparable to that for pCRELuc, as indicated in Figure 6.6C. However the mechanisms involved in the regulation of the pGL2-promoter in response to IFN-γ, and hence the impact on ICER 4xCARE, are not clear. This construct was not considered a useful model for further co-transfection experiments and instead pCRELuc was utilised.

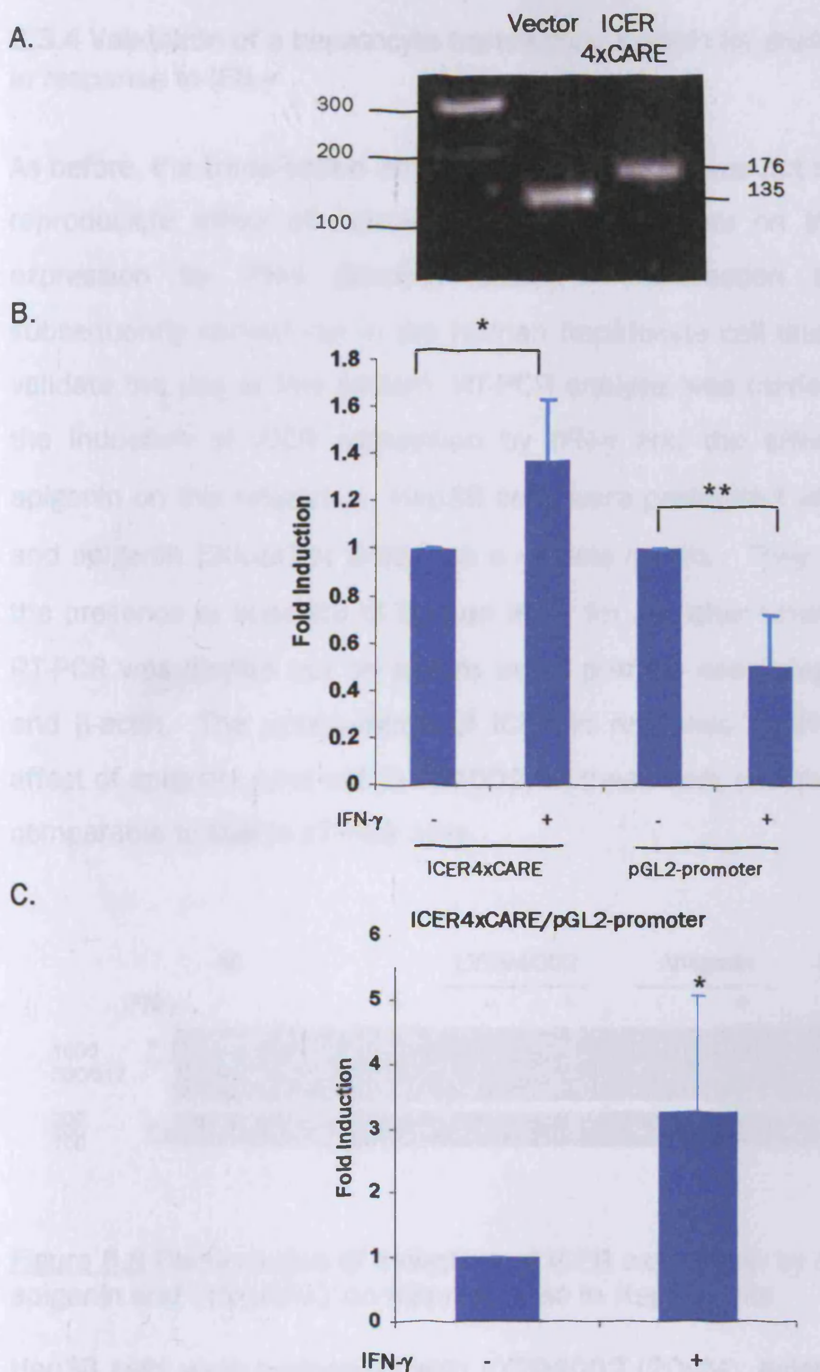


Figure 6.7 Cloning of a region of the ICER promoter into a luciferase reporter vector and the effect of IFN-γ

PCR was carried out using primers GL1 and GL2 on maxi prep DNA for empty pGL2-Promoter vector and the ICER 4xCARE construct. Products were resolved by agarose gel electrophoresis (A). U937 cells were transfected with the pGL2-Promoter expression vector and ICER4xCARE and treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration and presented as the mean (±SD) (B). Relative counts for ICER4xCARE were normalised to those for pGL2-promoter and presented as the mean fold induction (±SD) from four independent experiments. *$P<0.05$; **$P<0.01$.

6.3.4 Validation of a hepatocyte transfection system for studies of ICER expression in response to IFN-γ

As before, the transfection efficiency of U937 cells was not sufficient to observe a reproducible effect of dominant negative inhibitors on the induction of ICER expression by IFN-γ (Section 3.5.4). Transfection based studies were subsequently carried out in the human hepatocyte cell line, Hep3B. In order to validate the use of this system, RT-PCR analysis was carried out to demonstrate the induction of ICER expression by IFN-γ and the effect of LY294002 and apigenin on this response. Hep3B cells were pretreated with LY294002 (20μM) and apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of human IFN-γ for 3h, after which RNA was extracted. RT-PCR was carried out as before using primers specifying for human ICER I/II and β-actin. The upregulation of ICER in response to IFN-γ and the inhibitory effect of apigenin (and not LY294002) in these cells is shown in Figure 6.8 to be comparable to that in J774.2 cells.

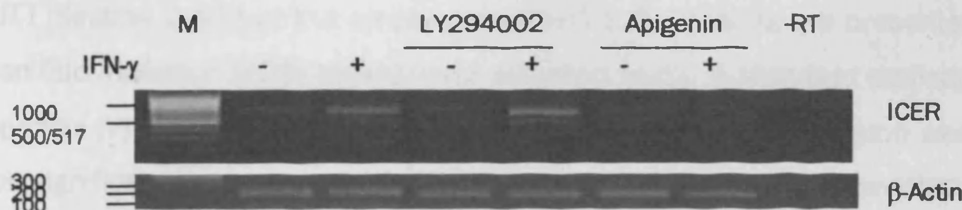


Figure 6.8 Confirmation of induction of ICER expression by IFN-γ and the effect of apigenin and LY294002 on this response in Hep3B cells

Hep3B cells were pretreated with LY294002 (20μM), apigenin (20μM) or DMSO as a vehicle control. They were then cultured in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against human ICER I/II and β-actin. The amplification products were analysed by agarose gel electrophoresis. The size of the DNA markers are indicated. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using vehicle-treated RNA). Results shown are representative of two independent experiments.

6.3.5 Autoregulation of the ICER promoter

As previously reported, IFN-γ induces the expression of ICER maximally following 2-6h stimulation (Figure 3.2). For treatment periods longer than this, the levels of ICER mRNA decline. This corresponds to the role of ICER in the regulation of cyclic processes such as the mammalian circadian rhythm (Link *et al.* 2004, Maronde *et al.* 1999, Foulkes *et al.* 1997). As the ICER promoter is regulated through tandem CRE-like elements, it is likely that the cyclic nature of ICER expression is due to autoregulation of the promoter, demonstrated in several studies (De Cesare and Sassone-Corsi 2000, Shaywitz and Greenburg 1999, Della-Fazia *et al.* 1997, Molina *et al.* 1993). To demonstrate this mechanism in the regulation of pCRELuc, ICER I was overexpressed and the effect on expression from pCRELuc analysed.

As described in Section 3.5.4, Hep3B cells were transfected with the luciferase reporter construct pCRELuc and the response to IFN-γ treatment (6h) analysed. The reporter construct was co-transfected with an expression plasmid encoding ICER I (Section 2.4.5) or the empty vector pcDNA3. Results are presented as the mean fold induction (±SD) (basal level assigned as 1). A standard statistical t-test (Appendix IV) was performed on the data and changes in expression were found to be significant to the level indicated. Overexpression of ICER prevented the IFN-γ-mediated activation of the pCRE-Luc promoter (as well as reducing basal levels of reporter expression) as shown in Figure 6.9, confirming that ICER is involved in the suppression of CREB-mediated signalling and that ICER is also likely to downregulate its own expression.

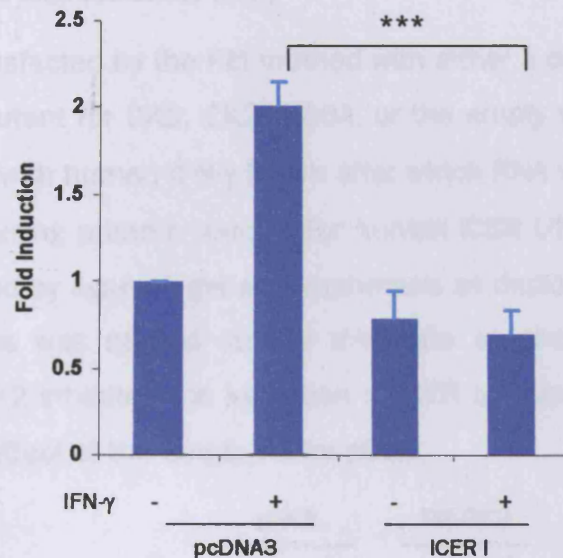


Figure 6.9 Inhibition of IFN-γ-mediated induction of pCRELuc activity by overexpression of ICER I

Hep3B cells were co-transfected with pCRELuc and a expression plasmid for ICER I or the vector pcDNA3. Transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration and presented as the mean fold induction (±SD) in response to IFN-γ, in relation to basal expression (assigned as 1), from three independent experiments. *** P<0.001.

6.4 CONFIRMATION OF THE INVOLVEMENT OF CK2 IN THE INDUCTION OF ICER EXPRESSION THROUGH USE OF A DOMINANT NEGATIVE CONSTRUCT

While use of the inhibitor apigenin has indicated a role for CK2 in the regulation of ICER gene expression by IFN-γ, a dominant negative construct specifying for a mutant form of CK2α was used to confirm the requirement for this kinase. Two approaches were used: the transfection of dominant negative CK2 followed by RT-PCR analysis of endogenous ICER I/II mRNA expression; and the co-transfection of the dominant negative construct with pCRELuc.

6.4.1 RT-PCR analysis of the effect of a dominant negative inhibitor of CK2 on the induction of ICER gene expression by IFN-γ

Hep3B cells were transfected by the PEI method with either a plasmid encoding a dominant negative mutant for CK2, CK2αK68A, or the empty vector pSG5 Cells were then stimulated with human IFN-γ for 3h after which RNA was extracted. RT-PCR was carried out using primers specific for human ICER I/II and β-actin. The products were resolved by agarose gel electrophoresis as depicted in Figure 6.9A. Densitometric analysis was carried out for this data as shown (Figure 6.9B). Dominant negative CK2 inhibited the induction of ICER I/II expression by IFN-γ in comparison with the effect of the empty vector pSG5.

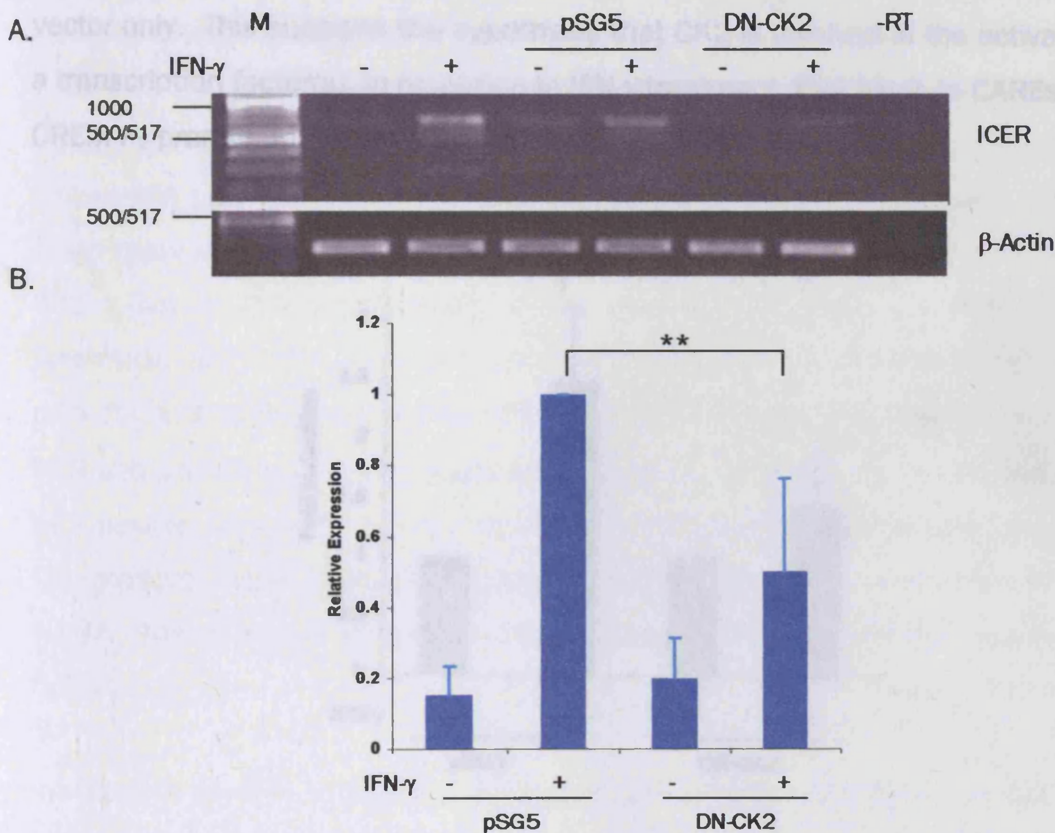


Figure 6.10 Inhibition of IFN-γ-mediated induction of ICER expression by dominant negative CK2

Hep3B cells were transfected with a dominant negative construct for CK2 or the vector pSG5. Transfected cells were incubated in the presence or absence of IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against human ICER I/II and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis from four independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using untreated RNA). **P<0.01.

6.4.2 Effect of a dominant negative inhibitor of CK2 on the activation of pCRELuc by IFN-γ

Co-transfection of pCRELuc and a dominant negative inhibitor of CK2 (CK2α-K68A) or pSG5 in Hep3B cells was carried out by the PEI method as before (Section 3.5.4) and cells were treated with IFN-γ for 6h. Results are presented as the mean fold induction (±SD) (basal level assigned as 1). A standard statistical t-test (Appendix IV) was performed on the data and changes in expression were found to be significant to the level indicated. Co-transfection with dominant negative CK2 led to an inhibition of the induction of reporter gene expression from pCRELuc by IFN-γ compared with co-transfection with the empty plasmid vector only. This supports the hypothesis that CK2 is involved in the activation of a transcription factor(s), in response to IFN-γ treatment, that binds to CAREs in the CREM P_2 promoter (Figure 6.11).

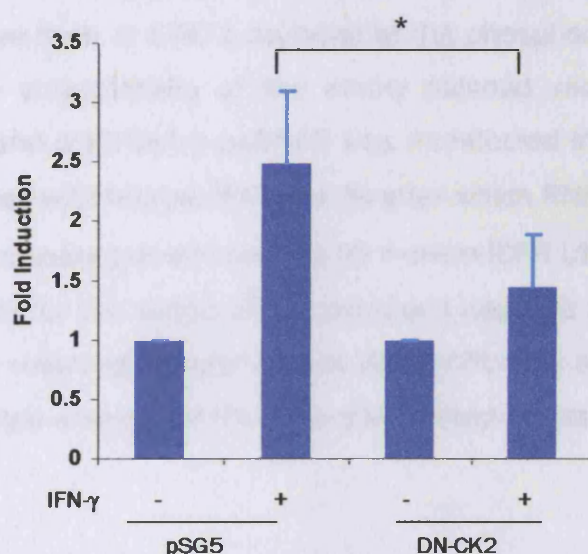


Figure 6.11 Inhibition of IFN-γ-mediated induction of ICER expression and pCRELuc activity by dominant negative CK2

Hep3B cells were co-transfected with pCRELuc and a plasmid construct specifying for a dominant negative mutant of CK2. Transfected cells were treated with IFN-γ for 6h after which cell extracts were prepared for luciferase reporter activity assay. Relative counts were normalised to protein concentration and presented as the mean fold induction (±SD) in response to IFN-γ, in relation to basal expression (assigned as 1) from four independent experiments. *$P<0.05$.

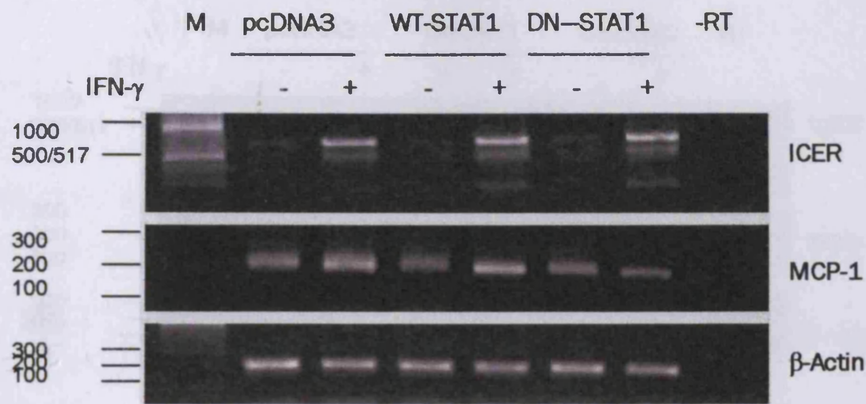
6.5 Role of the JAK-STAT Pathway in the Regulation of ICER Gene Expression by IFN-γ

Previous studies in our laboratory have found that the specific JAK2 inhibitor, AG490, does not suppress the stimulation of ICER gene expression by IFN-γ (Figure 4.2) (Mead *et al.* 2003). The activation of CK2 by IFN-γ, shown to be necessary for the induction of ICER expression in response to the cytokine, was also unaffected by pre-treatment with this inhibitor (Evans, S., personal communication).

To further investigate the role of the JAK-STAT pathway in the regulation of ICER, dominant negative forms of JAK1, JAK2 and STAT1 were transfected in Hep3B cells and the effect on the induction of ICER expression by IFN-γ determined through RT-PCR analysis. Hep3B cells were transfected by the PEI method as before with plasmids encoding dominant negative mutants of JAK1 and JAK2 and a dominant negative form of STAT1 mutated at the phosphorylation site Tyrosine 701. Due to the unavailability of the empty plasmid vectors used in these constructs (pRK5 and pRC/CMV), pcDNA3 was transfected in control cells. Cells were then stimulated with human IFN-γ for 3h after which RNA was extracted. RT-PCR was carried out using primers specific for human ICER I/II, β-actin and MCP-1 as a positive control for the action of the dominant negative mutants (Table 2.5). The products were resolved by agarose gel electrophoresis as depicted in Figure 6.12A. Densitometric analysis of the data was carried out as presented in Figure 6.12B.

Inactivation of each of these components (JAK1, JAK2 and STAT1) did not inhibit the induced expression of ICER mRNA by IFN-γ in comparison to an effect on the induction of MCP-1 by the cytokine. This suggests that the IFN-γ-mediated regulation of ICER expression occurs through a CK2-dependent mechanism independent of the JAK-STAT pathway.

A.



B..

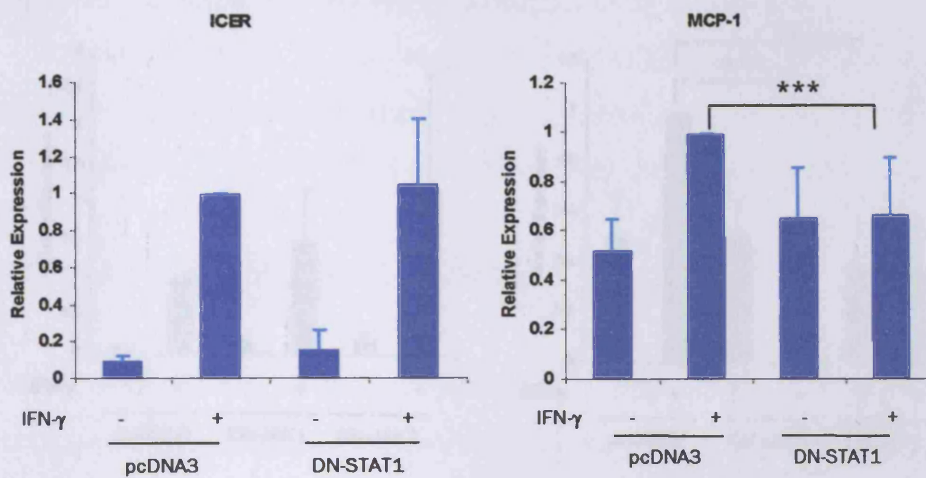
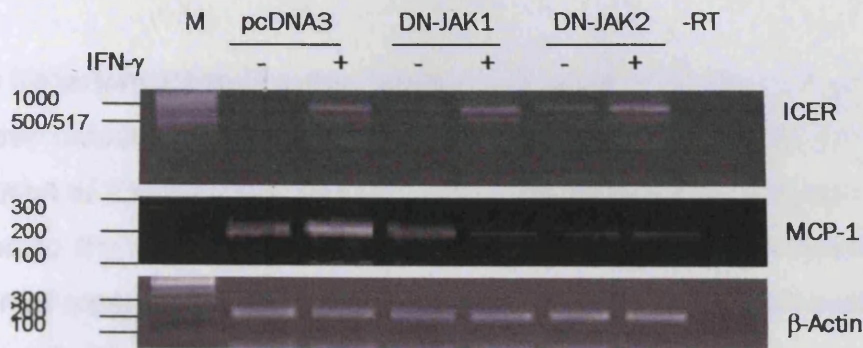


Figure 6.12 Effect of a dominant negative inhibitors of STAT1 on the IFN-γ-mediated induction of ICER expression

Hep3B cells were transfected with DN-STAT1 (Y701F), WT-STAT and pcDNA3. Transfected cells were exposed to IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against human ICER I/II and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis from three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using pcDNA3-transfected RNA). ***P<0.001.

A.



B..

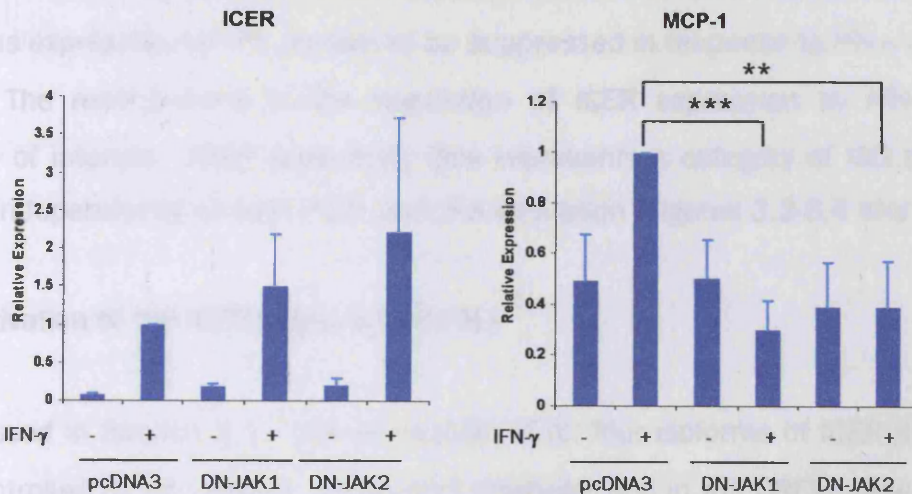


Figure 6.13 Effect of dominant negative inhibitors of JAK1 and JAK2 on the IFN-γ-mediated induction of ICER expression

Hep3B cells were transfected with DN-JAK1, DN-JAK2 and pcDNA3. Transfected cells were exposed to human IFN-γ for 3h. Total RNA was isolated and subjected to RT-PCR using primers against human ICER I/II and β-actin. The amplification products were analysed by agarose gel electrophoresis (A). The size of DNA markers are indicated. Panel B shows the relative expression (mean ±SD) normalised to the expression of β-actin as determined by densitometric analysis from three independent experiments. -RT denotes a reaction in which no reverse transcriptase was included for the cDNA synthesis step (using pcDNA3-transfected RNA). $^{**}P<0.01$; $^{***}P<0.001$.

6.6 DISCUSSION

ICER is an important transcriptional repressor of genes regulated through CREs. The dramatic induction of ICER mRNA expression by IFN-γ leads to speculation that the action of ICER may be involved in the suppression of a number of genes in response to the cytokine. In particular, a number of genes involved in the progression of atherosclerosis are downregulated by IFN-γ treatment such as SR-A, VLDL-R, and LRP (Schulz *et al.* 2003, Kosaka *et al.* 2001, LaMarre *et al.* 1993, Geng and Hansson 1992). The overexpression of ICER has been demonstrated to reduce the expression of LPL, known to be suppressed in response to IFN-γ (Mead 2002). The mechanisms in the regulation of ICER expression by IFN-γ are therefore of interest. ICER potentially also represents a category of ISG that is induced independently of both PI3K and JAK activation (Figures 3.2-3.4 and 4.2).

6.6.1 Activation of the ICER promoter by IFN-γ

As discussed in Section 1.11 the expression of all four isoforms of ICER (I, Iγ, II, IIγ) is controlled by an internal, alternative promoter (P_2) in the CREM gene. This promoter region contains four tandem CRE-related sequences known as CAREs as depicted in Figure 6.4. Use of the chemical inhibitor of transcription, actinomycin D, established that IFN-γ increased the transcription of ICER I/II mRNA in macrophages and that the response was not mediated by an increase in mRNA stability. A luciferase reporter construct (pCRELuc), in which expression of the luciferase gene is controlled by a TATA box linked to four CRE consensus enhancer elements, was used to model the response of the P_2 promoter regulating ICER expression. Transfection of this construct in U937 cells, differentiated in the presence of PMA, demonstrated IFN-γ-inducible regulation from a CRE-regulated promoter.

A reporter construct based on the pGL2-Promoter plasmid and regulated by a 63bp region of the ICER promoter containing four CAREs (ICER4xCARE), was generated to determine if the regulation by IFN-γ corresponds to that of pCRELuc. The ICER4xCARE construct, and the empty vector pGL2-Promoter, were transfected into U937 cells treated with IFN-γ as for the pCRELuc reporter construct. A statistically significant increase in reporter gene expression from ICER 4xCARE was stimulated by IFN-γ despite a corresponding decrease in the activity of the empty pGL2-promoter. A comparble induction in reporter gene expression from ICER 4xCARE and pCRELuc was observed when counts were normalised to those for the empty vector.

It is possible that the difference in expression from pCRELuc and ICER 4xCARE is due to specific differences in the functional sequence elements within the two promoters. A comparison of the pCRELuc and ICER4xCARE sequence highlights these differences (Figure 6.4B). Of the four CAREs, only CARE3 matches the CRE consensus sequence and these differences may only be tolerated within the context of the ICER promoter, allowing for the maximal upregulation by IFN-γ. The spacing between the four elements may also be crucial and only allow IFN-γ-mediated regulation in a context specific manner. An additional element may be present in the full length promoter that is vital for maximal IFN-γ-mediated upregulation from the CAREs but not the CRE consensus. Generation of a luciferase reporter containing a longer section of the ICER promoter may help to resolve this issue. It is likely however, that the nature of the minimal promoter elements in the vectors pLuc-MCS and pGL2-Promoter is responsible for the difference in pCRELuc and ICER 4xCARE activation by IFN-γ. Cloning the ICER 4xCARE promoter sequence into the pLuc-MCS plasmid may reduce this effect.

However for the purposes of this investigation the pCRELuc promoter was considered to be an adequate model for this response. Overexpression of ICER prevented the IFN-γ-mediated activation of the pCRE-Luc promoter as well as reducing basal levels of reporter expression (Figure 6.9). This indicates that the cyclic nature of ICER mRNA expression in response to IFN-γ, as demonstrated by RT-PCR analysis (Figure 3.2), is likely to be due to autoregulation of the promoter.

The autoregulation of ICER is also important in the regulation of cyclic processes such the mammalian circadian rhythm (Link *et al.* 2004, Maronde *et al.* 1999, Foulkes *et al.* 1997). In the absence of the empty vector for the pCRELuc construct (pLuc-MCS) this also allowed confirmation that the effect of IFN-γ on the activation of reporter gene expression is due to regulation through CREs and not an alternative element in the plasmid backbone. Together these studies suggest that CREs represent a novel class of IFN-γ-responsive elements.

6.6.2 Role of CK2 in the regulation of ICER expression by IFN-γ

Previous work in our laboratory, regarding the expression of ICER in response to IFN-γ, utilised a number of pharmacological inhibitors. These included those acting on PKA, PKC, p38 MAPK, ERK, Ca-calmodulin-dependent protein kinase and JAK2. None of these compounds were found to affect signalling by IFN-γ in respect to the expression of ICER (Mead *et al.* 2003). In this study it was also demonstrated that the specific inhibitor of JNK activity, SP600125 also failed to inhibit the induction of ICER by the cytokine (Figure 6.2). Thus far the IFN-γ-mediated expression of ICER is unique in that only the CK2 inhibitor apigenin has been found to affect the reponse.

The activity of CK2 in J774.2 cells has been shown to be induced maximally following stimulation with IFN-γ for 2h. A CK2-mediated increase in the phosphorylation of CREB is also observed in response to IFN-γ treatment (Mead *et al.* 2003). Activated CREB, or another member of the CREB/CREM/ATF-2 subfamily, is thought to subsequently bind to the alternative P_2 promoter of the CREM gene to increase the production of the ICER transcript. The upregulation of ICER by IFN-γ in isolated primary human monocyte-derived macrophages was observed to a similar level to that in J774.2 cells, and found to be inhibited by apigenin, indicating that these findings are not a peculiar property of the J774.2 cell line (Figure 6.3).

The involvement of CK2 in the IFN-γ-mediated regulation of ICER was confirmed in this study by transfection of a dominant negative construct specifying for a mutant form of CK2α into cells of the human hepatoma cell line, Hep3B. Due to the difficulty of DNA transfection in macrophages with suitable efficiency, the Hep3B cell line was selected as a high efficiency transfection system for the work described. The use of this cell line was justified by the similarity of the IFN-γ response for ICER expression in these cells shown by RT-PCR analysis (Figure 6.8). Transfection of CK2αK68A reduced the induction of ICER by IFN-γ in comparison to that in cells transfected with the empty plasmid expression vector pSG5 (Figure 6.10).

The construct CK2αK68A was also co-transfected with the luciferase reporter pCRELuc. This led to an inhibition of the induction of reporter gene expression from pCRELuc by IFN-γ compared to co-transfection with the empty plasmid vector only (Figure 6.11). This supports the hypothesis that CK2 is involved in the activation of CREB, in response to IFN-γ treatment, and this subsequently binds to CRE-like elements in the P_2 promoter.

6.6.3 Role of the JAK-STAT pathway in the regulation of ICER expression by IFN-γ

At present the upregulation of ICER expression by IFN-γ has not been linked to the activation of STAT1 and no GAS sites are present in the ICER promoter. The JAK2 inhibitor, AG490 was not shown to inhibit the IFN-γ-mediated induction of ICER expression (Figure 4.2) or the activation of CK2 in response to the cytokine (Evans, S., personal communication). Inactivation of JAK1 and JAK2 by the transfection of dominant negative mutant constructs in Hep3B cells did not inhibit the induced expression of ICER mRNA by IFN-γ, in comparison to an effect on the induction of MCP-1 by the cytokine (Figure 6.12). This supports the hypothesis that the IFN-γ-mediated activation of CK2 occurs in a pathway independent of JAK signalling

Similarly, transfection of a dominant negative construct encoding a mutant form of STAT1α in Hep3B cells did not prevent the induction of ICER expression by IFN-γ (Figure 6.13). While CK2 was implicated in the regulation of STAT1 activity by co-transfection experiments (Section 4.3) and by analysis of STAT1 phosphorylation, the role of CK2 in the expression of ICER, through phosphorylation of CREB, may be entirely STAT1-independent. The regulation of ICER expression by IFN-γ may therefore provide a novel JAK-STAT-independent mechanism of IFN-γ signalling.

<u>CHAPTER SEVEN:</u>

GENERAL DISCUSSION

CHAPTER 7. GENERAL DISCUSSION

7.1 MECHANISMS IN THE REGULATION OF GENE EXPRESSION BY IFN-γ

The pleiotropic cytokine IFN-γ was among the first cytokines to be identified and the actions and downstream signalling mechanisms of this important inflammatory mediator have been extensively studied. Identification of the JAK-STAT pathway in relation to IFN-γ signalling has provided a paradigm for intracellular cytokine-activated signalling pathways. However aspects of IFN-γ-activated signalling remain unclear in relation to both JAK-STAT-dependent and independent mechanisms and warrant further study. IFN-γ functions in the pathology of a number of diseases (Section 1.3.2) and has a complex role in the development of atherosclerosis (Section 1.4.6). The work detailed in this study has used several different approaches in order to elucidate novel mechanisms in the regulation of gene expression by IFN-γ, with particular emphasis on identifying potential targets that may be of relevance to the treatment of atherosclerosis. The majority of this work was 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 is hoped that such studies will not only enhance our understanding of foam cell formation and atherosclerosis but, in the long term, may also lead to the identification of potentially novel targets for therapeutic intervention in the disease.

Experiments based around the use of pharmacological inhibitors of potential mediators of IFN-γ signalling, identified a selective role for PI3K in the regulation of the expression of certain genes in response to the cytokine. This was subsequently supported through the use of kinase assays, co-immunoprecipitation and in studies using dominant negative mutants. The work was also extended to the IFN-γ-mediated regulation of expression of a wider range of genes in macrophages through microarray analysis. In contrast to PI3K, CK2 was found to have a common role in the IFN-γ-mediated regulation of expression for all the genes selected for study. This role has also been supported by further experimentation. Two genes, MCP-1 and ICER, were chosen for extensive study

into the mechanisms involved in the induction of expression by IFN-γ with the aim of identifying pathways that may later be extended to the regulation of other genes by the cytokine.

7.1.1 Role of CK2 in IFN-γ-mediated regulation of gene expression

The CK2 inhibitor apigenin prevented the IFN-γ-mediated induction of all the genes initially selected for study (ICER, MCP-1 and SOCS-1) and of those subsequently investigated (IP-10, Mig, I-TAC and CCR2). This suggests that CK2 may have a common role in the regulation of gene expression by IFN-γ in macrophages. The activation of CK2 is maximal in response to 2h incubation with IFN-γ consistent with a primary function in the regulation of gene expression following stimulation with the cytokine (Mead *et al.* 2003). Previous studies in our laboratory have shown that this response is not attenuated by the pharmacological inhibitors of JAK2 and PI3K, AG490 and LY294002 respectively (Evans, S., personal communication). The activation of PKB in response to IFN-γ stimulation was also unaffected by apigenin indicating that distinct pathways function in the regulation of PI3K and CK2 activity by IFN-γ.

Transfection based assays, in Hep3B cells, confirmed that CK2 is involved in the regulation of ICER expression by IFN-γ and indicated that CK2 may be involved in both the basal and induced expression of MCP-1. An effect of apigenin and a dominant negative mutant of CK2 on basal expression can also be seen in some, but not all, RT-PCR experiments. CK2 is a constitutively active kinase and it is reasonable to suppose that it may have a role in constitutive gene expression. A role for CK2 in the regulation of basal transcriptional machinery has also been documented, for example in the activity of RNA polymerases II and III (Cabrigos *et al.* 2004, Hu *et al.* 2003, Llorens *et al.* 2003, Johnston *et al.* 2002).

An effect on the activity of STAT1 has also been demonstrated for CK2. Co-transfection of dominant negative CK2 reduced the IFN-γ-induced expression of a luciferase reporter construct regulated by tandem GAS elements (3xly6e). Apigenin also reduced the phosphorylation of STAT1 on Serine 727, necessary for

maximal activation. Possibly CK2 has a dual role in the constitutive expression of the genes studied and in the IFN-γ response. CRE-mediated effects of IFN-γ (*e.g.* ICER gene transcription) appear to require CK2 and STAT1-mediated responses may do also. In addition to cytokine-inducible activation, STAT1 has a role in the constitutive regulation of certain genes (Meyer *et al.* 2002, Chatterjee-Kishore *et al.* 2000). EMSA and ChIP analysis indicated that STAT1 binds constitutively to a GAS element in the proximal promoter of the MCP-1 gene, as well as being induced by IFN-γ, that may maintain a low basal level of expression. CK2 may have a role in the constitutive regulation of STAT1 that is increased upon IFN-γ-mediated activation of the kinase. CK2 is a pleiotropic kinase with a large number of identified and potential substrates, and is likely to have diverse roles in the regulation of many genes (Meggio and Pinna 2003). The possibility remains that CK2 is involved in a different mechanism for each of the genes studied.

7.1.2 Role of PI3K in IFN-γ-mediated regulation of gene expression

In contrast to CK2, PI3K appears a have a selective role in the IFN-γ-mediated regulation of gene expression in macrophages. Of the genes initially selected for investigation (MCP-1, SOCS-1 and ICER), the PI3K inhibitor LY294002 inhibited the induction of only MCP-1 gene expression by IFN-γ. The role of PI3K was supported in the regulation of MCP-1 expression through the use of a second inhibitor, wortmannin, and a plasmid construct specifying for a dominant negative form of the downstream effector of PI3K, PKB.

Extension of this line of experiment to the regulation of global macrophage gene expression by IFN-γ, through microarray analysis, also indicated a selective role for PI3K in these responses. Using a microarray representing genes associated with the progression of atherosclerosis, it was found that the expression of 50 of these genes was induced by IFN-γ, while the expression of 3 was suppressed. The genes that were upregulated included a large number of cytokines and chemokines. The IFN-γ-induced expression of 39 of the genes was inhibited in the presence of LY294002. It remains to be determined whether the large proportion of atherosclerosis-associated genes for which the regulation by IFN-γ was affected by the inhibitor could have relevance to the disease and potential therapies.

The induction of the chemokines IP-10, Mig and I-TAC, and the receptor CCR2 by IFN-γ was also found to be mediated through a PI3K-dependent pathway. The mechanisms behind this selectivity have yet to be identified (discussed below). The function of PI3K in the induction of MCP-1 expression by IFN-γ has also been indicated in ECs opening the possibility that these findings may be extended to other cells of the atherosclerotic plaque.

The downstream effector of PI3K, PKB, has been shown in previous studies to be activated in response to IFN-γ treatment (Nguyen *et al.* 2001, Hwang *et al.* 2004, Navarro 2003). This was also demonstrated in this study in J774.2 macrophages. PKB was found to be involved in the induction of MCP-1 gene expression by IFN-γ and is likely to act as a downstream effector of PI3K in this response. The induction of PKB activity by IFN-γ was found to be dependent on JAK2. Co-immunoprecipitation experiments demonstrated an interaction between the PI3K subunit p85 and JAK2 suggesting that the activation of JAKs by IFN-γ may be directly responsible for the activation of PI3K and PKB.

Several previous studies implicate the activation of PI3K in the regulation of STAT1 by IFN-γ (Choudhury 2004, Hwang *et al.* 2004, Nguyen *et al.* 2001). This was supported, as a potential mechanism in macrophages, by the data presented. A dominant negative form of PKB reduced IFN-γ-induced expression from the luciferase reporter construct 3xly6e and LY294002 inhibited the phosphorylation of STAT1 on Serine 727. Choudhury *et al.* (2004) have also presented data showing that LY294002 affected the stimulation of MCP-1 expression by IFN-γ in mesangial cells. They found that LY294002 reduced IFN-γ-induced phosphorylation of STAT1 at Serine 727 and GAS-activity but that it did not affect IFN-γ-induced binding to a consensus STAT1 element *in vitro*. This suggests that PI3K regulates the activation potential of STAT1 in a manner that does not alter the DNA binding activity. However, although PI3K is involved in the activation of STAT1, LY294002 does not inhibit all IFN-γ-regulated genes including those known to be induced through the JAK-STAT pathway such as SOCS-1.

An important factor in the selective requirement for PI3K in IFN-γ-signalling may be in the control of the interaction of STAT1 with other factors such as Sp1, shown to be inducible by IFN-γ. The role of Serine 727 in the activity of STAT1 is unclear at present but appears to be involved in the recruitment of coactivators such as MCM5 and BRCA1 (Ouchi *et al.* 2000, Zhang *et al.* 1998). It is possible that it may also function in interactions with other transcription factors including Sp1 either directly or through such coactivators. Such interactions are likely to be specific to the regulation of particular genes and potentially mediated by the proximity of the appropriate binding sites in the promoter.

Alternatively the selective action of PI3K may be to alter the affinity of STAT1 for specific promoter sequences. Naik *et al.* (1997) have demonstrated that an IFN-γ-activated STAT1 complex (gamma response factor (GRF)) binds to an element in the human ICAM-1 promoter but not to the classical GAS element. Interestingly, while dominant negative inhibition of PKB reduced the activation of a GAS-regulated promoter by IFN-γ, the induction of ICAM-1 in response to the cytokine was not affected by the presence of LY294002. This may be due to a similar, sequence specific mechanism.

7.1.3 Regulation of MCP-1 gene expression by IFN-γ

The expression of MCP-1 has been shown by Gil *et al.* (2001) to be induced by IFN-γ in both wild type and STAT1-deficient macrophages. However, STAT1 has been shown through several independent studies to be involved in the IFN-γ-mediated upregulation of MCP-1 in a variety of cell types but not in macrophages (Zhou *et al.* 2001, Valente *et al.* 1998, Zhou *et al.* 1998). In this study, both potential STAT1-dependent and -independent pathways in the regulation of MCP-1 expresssion by IFN-γ were investigated.

Initial work identified a role for CK2 and PI3K through the use of the pharmacological inhibitors, apigenin and LY294002. The downstream effector of PI3K, PKB was subsequently found to also be necessary for the response confirmed with the use of a dominant negative construct. Transfection based

studies using a dominant negative mutant of CK2α indicated that CK2 may have a role in both the constitutive and induced expression of MCP-1. Transfection of a dominant negative form of STAT1 inhibited the activation of the MCP-1 promoter by 46.5% in comparison to an inhibition of 89% of the GAS regulated construct 3xly6e. CK2 and PKB dominant negative mutants inhibited the MCP-1 response to a similar degree. In contrast, a dominant negative form of JAK2 almost completely prevented the activation of the MCP-1 promoter in response to IFN-γ and dominant negative JAK1 to a slightly lesser extent. It is possible that alternative IFN-γ signalling pathways for the regulation of MCP-1 expression may exist, but that all mechanism are dependent on JAK kinase activity. Both CK2 and PI3K were shown to regulate the activity of STAT1 binding activity through the phosphorylation of STAT1 at Serine 727, however this does not rule out the possibility that one or both of these kinases is also involved in a STAT1-independent mechanism.

Promoter binding analysis revealed an increase in STAT1 binding to a GAS element of the MCP-1 promoter with IFN-γ. However constitutive binding of STAT1 was also observed. It is possible that CK2 may have a role in the constitutive activity of STAT1 and that this is responsible for the reduction in the basal activity of the MCP-1 promoter observed in co-transfection studies using dominant negative CK2. Sp1 was also shown to bind constitutively to a GC box in the proximal MCP-1 promoter. CK2 has been shown in our laboratory to immunoprecipitate with Sp1 and independent studies have demonstrated the phosphorylation of Sp1 by CK2 (Armstrong *et al.* 1997, Evans, S., personal communication). An IFN-γ-inducible interaction was demonstrated between Sp1 and STAT1 and the two factors may co-operate to regulate the expression of MCP-1 as previously suggested by Look *et al.* (1995) in the regulation of ICAM-1. Although the phosphorylation of Sp1 by CK2 has previously been shown to decrease Sp1 binding to the LPL promoter (Hughes et al. 2002, Evans, S., personal communication), in the context of the MCP-1 promoter, this may also have a role in the IFN-γ-inducible interaction with STAT1.

7.1.4 Regulation of ICER gene expression by IFN-γ

The expression of ICER is regulated through an internal promoter of the CREM gene (P_2) that contains four tandem CRE-like elements known as CAREs. It has been shown previously in our laboratory that CK2 is activated by IFN-γ and subsequently phosphorylates CREB. A luciferase reporter plasmid, pCRELuc, regulated by four CRE consensus sites and used to model the ICER promoter, was upregulated by IFN-γ in U937 cells. This activity was inhibited by the overexpression of ICER I. A significant induction was also observed in the expression of a reporter gene regulated by a section of the CREM P_2 promoter containing four CRE-like elements (CAREs). These studies identify CREs as a novel class of IFN-γ responsive element that merits further investigation.

Thus far CK2 is the only signalling mediator for which a role has been established in the regulation of ICER expression by IFN-γ. The IFN-γ-mediated upregulation of ICER is not suppressed by the JAK2 inhibitor AG490 in J774.2 cells or by transfection of dominant negative forms of JAK1, JAK2 or STAT1 in Hep3B cells. The regulation of ICER expression by IFN-γ therefore appears to represent a novel JAK-STAT independent signalling mechanism. However, it still remains to rule out the possibility that members of the JAK or STAT families of proteins are acting in a functionally redundant mechanism in the IFN-γ-mediated induction of ICER expression. It is currently unclear which other effectors of IFN-γ signalling may be involved in the regulation of ICER by IFN-γ, if any.

7.1.5 Differential pathways in the IFN-γ-mediated regulation of gene expression

In course of this study three differential pathways have been identified in the IFN-γ-mediated regulation of macrophage gene expression. The regulation of the expression of the majority of genes by IFN-γ appears to be dependent on the action of JAKs. The activation of PI3K and PKB is mediated through JAKs and has a role in the modulation of STAT1 activity through the phosphorylation of Serine 727. However an important observation, made in this study and others (Hwang *et al.* 2004), is that not all genes that are regulated by IFN-γ require PI3K for this

response. This suggests the division of ISGs, as depicted in Figure 7.1, into those for which the IFN-γ response is "PI3K-dependent" (*e.g.* MCP-1) and those regulated through the classical "JAK-STAT" pathway (*e.g.* SOCS-1). Initial observations indicated an inverse relationship between STAT1- and PI3K-dependence in the induction of MCP-1 and SOCS-1 expression by IFN-γ based on the findings by Gil *et al.* (2001). However there does not appear to be an obvious correlation between PI3K-dependence and STAT1-dependence as the chemokines Mig and IP-10, the expression of which is regulated by IFN-γ in a PI3K-dependent manner, have been shown by in the study by Gil *et al.* (2001) to be induced in wild type cells only (Gil *et al.* 2001, Ramana *et al.* 2002).

The third pathway is that involved in the stimulation of ICER expression in response to IFN-γ. This requires the JAK-independent activation of CK2 that leads to the phosphorylation and activation of CREB. CREB, or other members of the CREB/CREM/ATF-2 subfamily, may also therefore be involved in the expression of other genes stimulated by IFN-γ. The activation of CK2 may also lead to the activation of other transcription factors (*e.g.* NF-κB, AP-1) involved in the regulation of a diverse range of genes. CK2 may function in the constitutive and IFN-γ-induced expression of many ISGs through regulation of STAT1-binding potential or interaction with other factors such as Sp1. Alternatively, it is possible that the role of CK2 in the regulation of the majority of these genes is mediated through actions on basal transcriptional machinery such as RNA polymerases (Cabrigos *et al.* 2004, Hu *et al.* 2003, Llorens *et al.* 2003, Johnston *et al.* 2002).

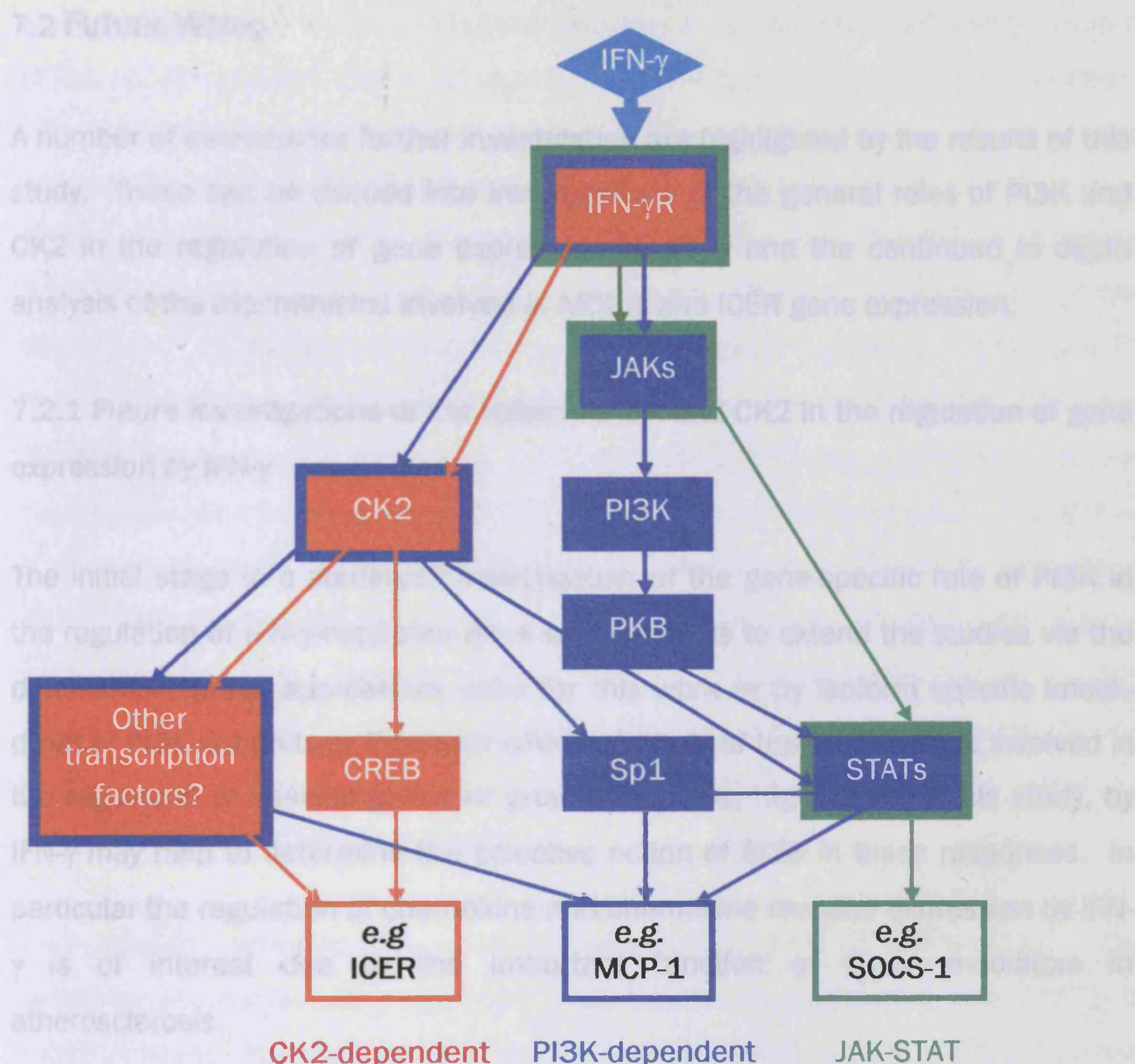


Figure 7.1 Differential pathways in the IFN-γ-mediated regulation of gene expression

The "CK2-dependent" pathway (red) responsible for the induction of ICER expression in response to IFN-γ involves the JAK-independent activation of CK2 leading to the phosphorylation of CREB or a related factor. Other transcription factors may also be activated by CK2-dependent phosphorylation and regulate the expression of other genes. The "PI3K-dependent" pathway (blue) potentially regulates the expression of a number of ISGs including MCP-1. JAK-dependent activation of PI3K leads to the activation of PKB and the DNA-binding of STATs. CK2 may also have a role in the regulation of these genes. The classical "JAK-STAT" pathway (green) illustrated is used to describe a mechanism in which STAT1 is activated through JAKs in a PI3K-independent manner, involved in the IFN-γ-mediated regulation of genes such as SOCS-1. For simplicity, the potential role of CK2 in this pathway has been ommitted.

Abbreviations: CK2, casein kinase 2; CREB, CRE-binding protein; ICER, inducible cAMP early repressor; IFN-γ, interferon-γ; IFN-γR, IFN-γ receptor; JAK, janus kinase; MCP-1, monocyte chemoattractant protein-1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; SOCS-1, suppressor of cytokine signalling-1; STAT, signal transducer and activator of transcription

7.2 FUTURE WORK

A number of avenues for further investigation are highlighted by the results of this study. These can be divided into investigations of the general roles of PI3K and CK2 in the regulation of gene expression by IFN-γ and the continued in depth analysis of the mechanisms involved in MCP-1 and ICER gene expression.

7.2.1 Future investigations of the roles of PI3K and CK2 in the regulation of gene expression by IFN-γ

The initial stage in a continued investigation of the gene-specific role of PI3K in the regulation of IFN-γ-mediated gene expression, is to extend the studies via the dominant negative approaches used for this work or by isoform specific knock-down of PI3K subunits or PKB with siRNA. A study of the mechanisms involved in the regulation of specific genes or groups of genes, highlighted in this study, by IFN-γ may help to determine the selective action of PI3K in these responses. In particular the regulation of chemokine and chemokine receptor expression by IFN-γ is of interest due to the important function of these mediators in atherosclerosis.

PI3K-dependence may be dictated by the presence of certain elements in the promoter of target genes and require interactions such as that between Sp1 and STAT1. IFN-γ-inducible interactions between these factors may be mediated through the action of PI3K or CK2. Further studies of protein-protein interactions, by co-immunoprecipitation or a mammalian two-hybrid assay, could be carried out in the presence of inhibitors for these kinases. Glutathione S-transferase (GST)-fusion proteins could be used to identify the domains required for interaction. The GST domain would be linked to domains of a mediator of interest and the fusion protein incubated with treated protein extracts. Complexes could then be easily purified and the interacting protein identified.

The function of PI3K and CK2 in the regulation of STAT1 activity should also be studied in more detail. PKB may potentially phosphorylate STAT1 at Serine 727

analysis of the functional interactions between these proteins and demonstration of the ability of active PKB to phosphorylate STAT1 in an *in vitro* kinase reaction. The role of Serine 727 in the regulation of STAT1 activity is unclear at present but appears to be involved in the recruitment of coactivators such as MCM5 and BRCA1 (Ouchi *et al.* 2000, Zhang *et al.* 1998). It is possible that it may also function in interactions with other transcription factors including Sp1 either directly or through such coactivators. Expression of a mutant form of STAT1 lacking the Serine 727 phosphorylation site only partially inhibited the IFN-γ-stimulated activation of the 3xly6e promoter. Phosphorylation of this site may not be required for the activation of all STAT1-mediated gene expression. The use of a mutant form of STAT1 lacking this phosphorylation site could indicate a differential requirement for Serine 727 phosphorylation in the regulation of gene expression by IFN-γ. Similarly, mice or cells with genetic mutations of this site could be generated to investigate these effects. PI3K and PKB may also be involved in the activation of STAT1 via the phosphorylation of other regulatory sites that are as yet undetermined.

CK2 similarly affected the phosphorylation of STAT1 at Serine 727. Co-immunoprecipitation did not show an interaction between CK2 and STAT1 in macrophages. This does not rule out the possibility that CK2 is involved in direct phosphorylation of STAT1 as this technique may not reveal reactions for which dimerisation is unstable or transient. Incubation of recombinant CK2 and STAT1 in an *in vitro* kinase reaction could be used demonstrate this action.

The activity of CK2 is regulated in an independent manner to PKB but these pathways may converge to regulate a common STAT1-kinase responsible for phosphorylation at Serine 727. Further studies should aim to identify a putative downstream effector. Other pathways that have been linked to the phosphorylation of STAT1 at Serine 727 in response to various mediators include those dependent on JNK, ERK, p38 and PKC (Kovarik *et al.* 1999). These kinases may also be involved in pathways mediated by PI3K or CK2. Preliminary studies have found that the induction of SOCS-1 expression by IFN-γ is inhibited in the presence of the JNK inhibitor SP600125 (Figure 6.2). The activation of JNK has previously been linked to CK2 kinase activity (Hilgard *et al.* 2004, Riga *et al.*

previously been linked to CK2 kinase activity (Hilgard *et al.* 2004, Riga *et al.* 2004, Fleming *et al.* 2000). Further studies may elucidate a common pathway involving these kinases in the cytokine-inducible regulation of STAT1.

The mechanism involved in the IFN-γ-stimulated activation of CK2 is undetermined. While it has been shown that this response occurs independently of JAKs it is likely to require the activation of IFN-γR and the binding of an upstream kinase, or CK2 itself, to the receptor. The necessity of IFN-γR should be confirmed in cells lacking this receptor. Subsequently, mutational analyses could be carried out in order to determine which regions of the receptor are required to mediate the activation of CK2 by IFN-γ. Co-immunoprecipitation or mammalian two-hybrid assays could be used to demonstrate the binding of either CK2 itself or putative upstream kinases to IFN-γR. Immunofluorescent staining could allow the determination of whether CK2 is recruited to the cell membrane upon stimulation with IFN-γ. It is unclear whether cytokine-inducible CK2 activity is mediated through the phosphorylation and activation of individual subunits of CK2 or by promoting assembly of the CK2 heterotetramer. This issue should also be addressed in future studies through the use of phospho-peptide mapping of CK2 subunits and analysing subunit interactions by co-immunoprecipitation and mammalian two-hybrid assays.

The common mechanism or mechanisms by which CK2 is involved in the IFN-γ-inducible regulation of all the genes studied remains to be determined. While the activation of CK2 by IFN-γ suggests that it does have a role in cytokine-induced gene expression, as CK2 is constutitvely active, part of its function may be in the maintenance of basal expression for certain genes, as may be the case for MCP-1. The effect of a dominant negative form of CK2 on the basal promoter expression for other ISGs should therefore be analysed. Genome wide arrays could be used to determine the function of CK2 in both basal and IFN-γ-mediated transcriptional control.

7.2.2 Future investigations of the mechanisms of the IFN-γ-mediated induction of MCP-1 and ICER gene expression

One of the initial aims of this study was to investigate the potential role of a STAT1-independent signalling pathway in the regulation of MCP-1 gene expression by IFN-γ. Such a mechanism has not thus far been identified. However STAT3 can also be activated by IFN-γ and Ramana *et al.* (2005) have recently demonstrated that STAT3 may function in place of STAT1 in STAT1-deficient cells, at least for the regulation of SOCS-3 expression (Ramana *et al.* 2005, Qing *et al.* 2004). This may explain the finding of Gil *et al.* (2001) that the induction of MCP-1 expression by IFN-γ is unaffected in STAT1-null macrophages while in the cellular systems used in this study and others, STAT1 has been shown to be required. While in STAT1 knock-out cells STAT3 may act in place of STAT1, a dominant negative inhibitor such as that used here may also sequester active STAT3 through heterodimerisation and hence attenuate the response to IFN-γ (Ramana *et al.* 2005, Qing *et al.* 2004). Transfection based studies utilising a dominant negative mutant of STAT3, available in the laboratory, should help to resolve this issue.

Alternatively promoter elements in regions outside that studied (213bp upstream of transcriptional start site) may be involved in a STAT1-independent response. The role of putative NF-κB and C/EBP-β binding sites in the distal MCP-1 promoter is worthy of investigation, particularly as IFN-γ was shown to mediate a significant induction in the expression of a reporter gene regulated by consensus NF-κB and C/EBP-β binding elements. Other components of PI3K-dependent and CK2-dependent pathways in the IFN-γ-mediated induction of MCP-1 expression may also be identified through the use of pharmacological inhibitors, dominant negative and siRNA based approaches, kinase activity assays, promoter dissection, EMSA and ChIP analysis.

The regulation of ICER expression by IFN-γ is of particular interest as it represents a potentially novel mechanism of signalling in IFN-γ-mediated responses. Initial studies should be performed in cells lacking functional JAK1, JAK2 and JAK3 in

order to assess the possibility that these kinases are acting in a redundant manner in the regulation of ICER expression and CK2 activation by IFN-γ. A functional dissection of the ICER promoter should be carried out to identify binding sites for other transcription factors that may be involved in the IFN-γ response. EMSA and ChIP analysis could be used to confirm the binding of CREB, or related family members, to the region containing tandem CAREs and to show whether this is inhibited in the presence of apigenin. As CREB is likely to be phosphorylated directly by CK2 (Meggio and Pinna 2003) co-immunoprecipitation could be used to demonstrate an IFN-γ-inducible interaction between these proteins. The domains required for interaction could subsequently be studied through the use of 'pull-down' assays with GST-fusion proteins. If an interaction were shown, *in vitro* kinase assays should be carried out using CREB as a substrate for CK2. The experiments detailed have so far failed to reveal components of this signalling pathway in addition to CK2. Future studies will aim to identify such components through the use of pharmacological inhibitors, dominant negative and siRNA based approaches and kinase activity assays.

7.3 IMPLICATIONS FOR THE TREATMENT OF ATHEROSCLEROSIS

The chemokine MCP-1 is a proatherogenic factor (Section 1.6) and represents a good therapeutic target for the treatment of atherosclerosis. Previous studies have demonstrated that transfection of a N-terminal deletion mutant of the MCP-1 gene (7ND) in apoE-null mice inhibits both lesion formation and the progression of existing lesions, as well as increasing plaque stability (Inoue *et al.* 2002, Ni *et al.* 2001). The broad based inhibition of the action of chemokines has also been investigated as a potential treatment for inflammatory disease with some success (Johnson *et al.* 2004, Grainger and Reckless 2003). The findings in this study may highlight further mechanisms by which the inflammatory effects of MCP-1 may be reduced.

The identification of a common PI3K-dependent pathway in the regulation of chemokine expression by IFN-γ in macrophages suggests a beneficial, atheroprotective effect to the specific inhibition of PI3K signalling or the downstream effector PKB. This pathway may also function in ECs, which are

important in the production and secretion of chemokines in inflammation. PI3K-dependent signalling mechanisms are often activated in response to chemokine stimulation, and as such, the inhibition of PI3K activity has previously been suggested as a potential therapeutic strategy in inflammatory disease (Johnson *et al.* 2004) In LDL-R-null mouse models of atherosclerosis, deficiency in the PI3K subunit p110δ in bone marrow transplants, has been found to abrogate the development of the disease (Pinderski *et al.* 2005). Therapeutic strategies have been investigated for the treatment of certain tumors that involve the inhibition of PI3K-dependent signalling (Berrie 2001) and it is possible that these may be applied in atherosclerosis. The requirement of CK2 in IFN-γ-stimulated responses indicates that signalling through this kinase may also provide a target for therapeutic intervention in atherosclerosis. The dominant negative inhibition of CK2 activity has been shown to reduce the progression of the inflammatory disease glomerulonephritis in rats and similar mechanisms may operate in the pathology of atherosclerosis (Yamada *et al.* 2005).

Both CK2 and PI3K are important biological mediators with a diverse range of actions in normal cellular function. In particular, the multitude of actions and targets of CK2 have yet to be studied comprehensively. PI3K also has beneficial effects on the development of diabetes (Cho *et al.* 2001, Shepherd *et al.* 1998). Direct inhibition of these kinases therefore, while a promising therapeutic strategy, is likely to affect a number of cellular systems. However the studies reported may lead to the identification of factors that are specific to the inflammatory pathways that are exacerbated in atherosclerosis and as such provide good targets for therapeutic intervention.

From the work presented in this thesis it is clear that the regulation of gene expression by IFN-γ in macrophages is dependent on several signalling pathways. IFN-γ-dependent signalling and the target genes studied have important functions in the pathogenesis of atherosclerosis. A complete understanding of the molecular mechanisms involved will hopefully lead to the development of new therapeutic strategies to combat this disease.

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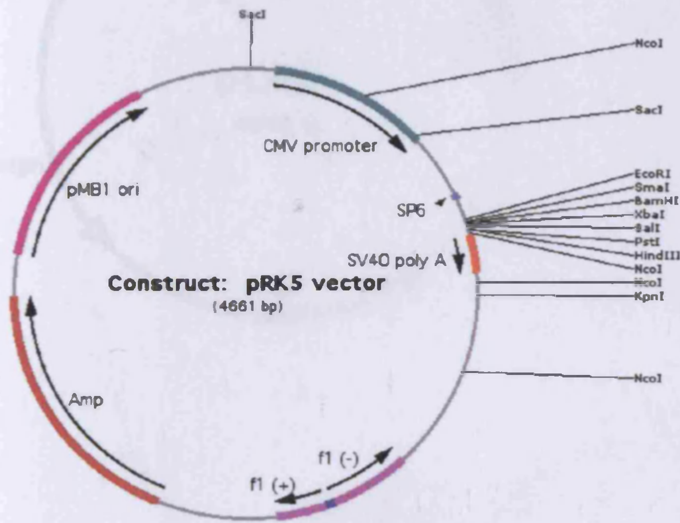
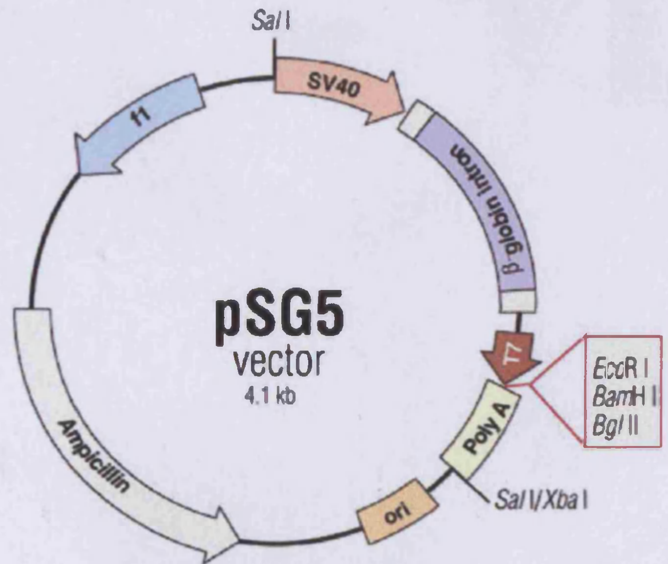
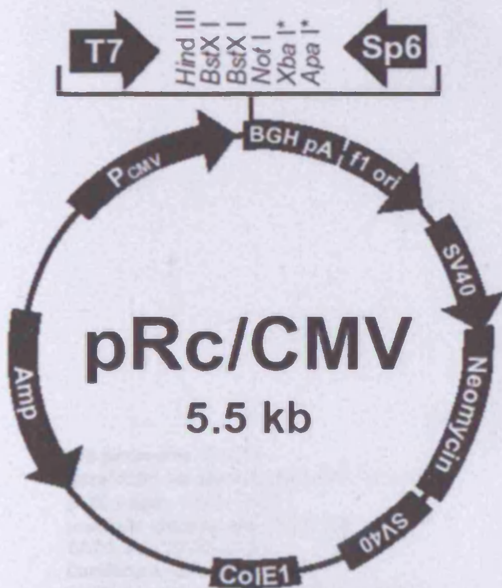
APPENDICES

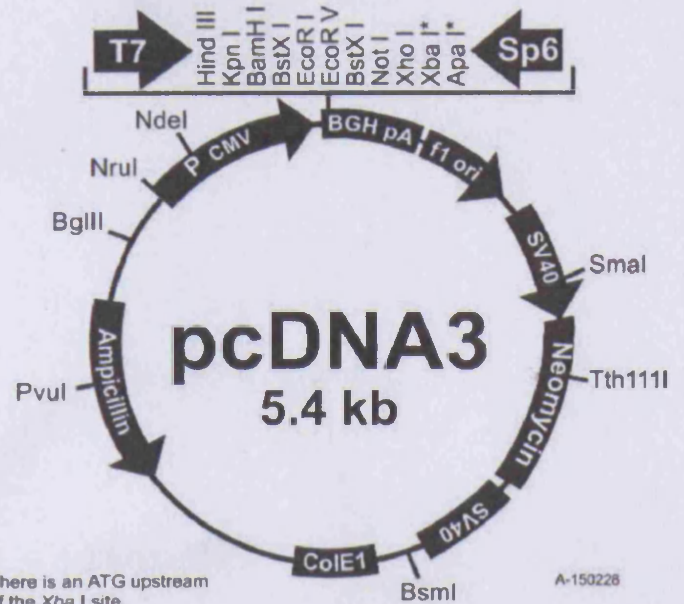
APPENDIX I

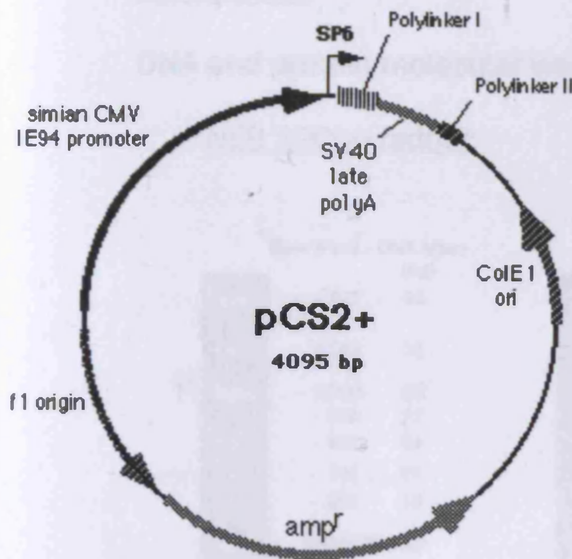
Plasmid vectors

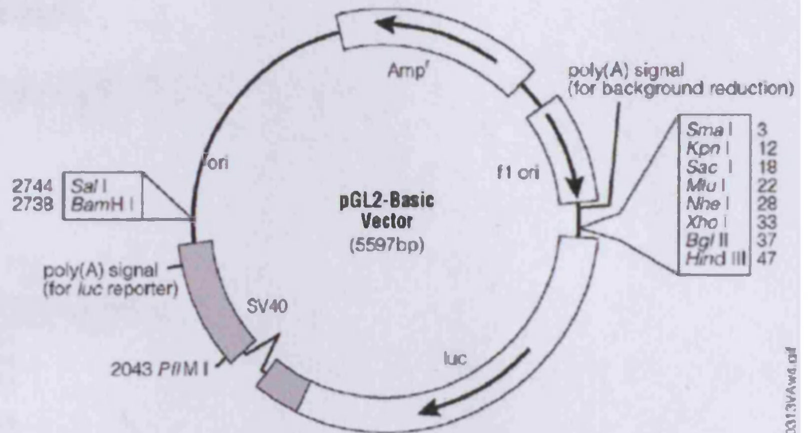


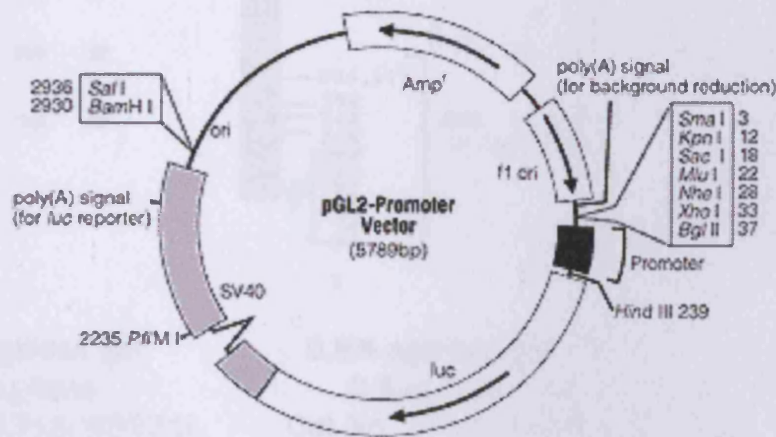


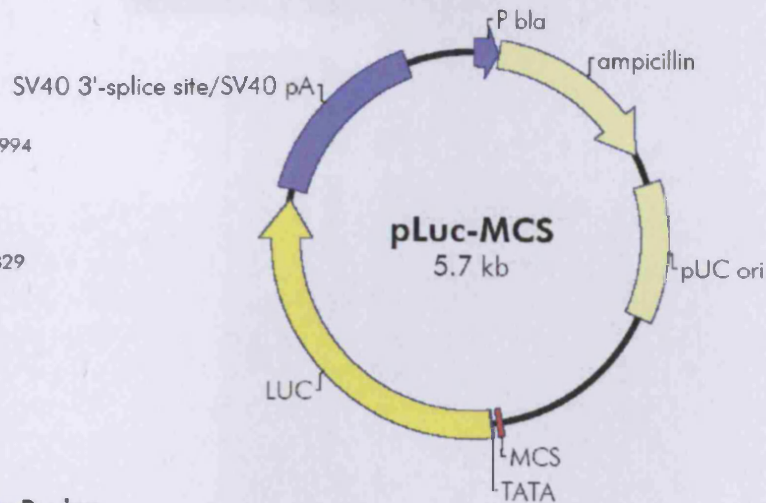












***bla* promoter** 13–134
ampicillin resistance (*bla*) ORF 137–994
pUC origin 1145–1810
multiple cloning site 2675–2701
TATA box 2725–2730
Luciferase ORF 2731–4428
SV40 3′-splice/SV40 polyA 4476–5329

pLuc-MCS Multiple Cloning Site Region
(sequence shown 2675–2730)

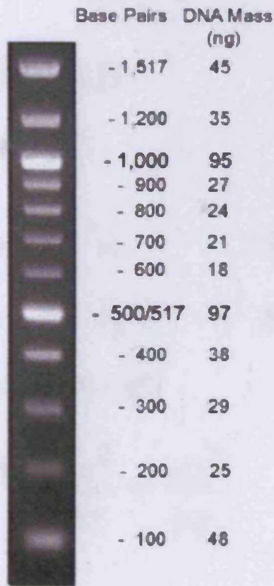
Hind III Srf I Sma I/Xma I Bgl II Xho I Sal I TATA box

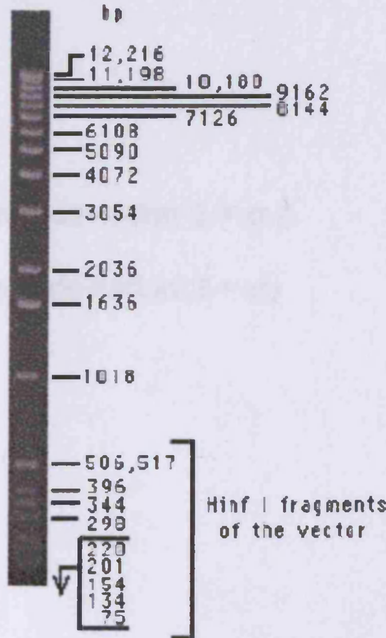
AAGCTTGCCCGGGCAGATCTCTCGAGGTCGACAGCGGAGACTCTAGAGGGTATATA

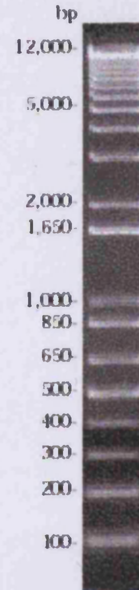
APPENDIX II

DNA and protein molecular weight markers

NEB 100bp ladder	Invitrogen 1kb ladder	Gibco 1kb plus ladder

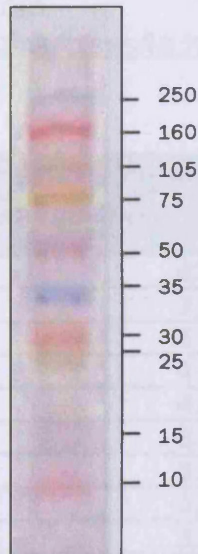






1.3% agarose gel	0.9% agarose gel	0.9% agarose gel
0.5μg/lane	0.5μg/lane	0.9μg/lane
Cat No. N3231S/N3231L	Cat No. 15615-016	Cat No. 10787-018

Amersham Full-Range Rainbow™
Molecular Weight Markers



12% SDS-PAGE gel
Cat. No. RPN800

APPENDIX III

Sequence of ICER 4xCARE insert in the pGL2-Promoter vector

GL1[TGTATCTTATGGTACTGTAACTGA]GCTAACATAACCCGGGTTTCAGTGAGCTGCACATTGATGGCAGTGA
TAGGCTGGTGACGTCACTGTGATGTCAGTGCTCCTCGAGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGT
AAAA[TGGAAGACGCCAAAAACATAAAGAA]GL2

APPENDIX IV

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 $(n_1 + n_2)-2$, where n_1 is the number of replicates of treatment 1, and n_2 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

APPENDIX V

Layout of gene specific oligonucleotides in the Oligo GEArray™ Mouse Atherosclerosis Microarray

Gapdh 1	Abca1 2	Ace 3	Adfp 4	Apoa1 5	Apoa2 6	Apoa4 7	Apob 8
Apoe 9	Bax 10	Bcl2 11	Bcl2a1a 12	Bcl2l1 13	Bid 14	Birc3 15	Ccl11 16
Ccl2 17	Ccl20 18	Ccl5 19	Ccr1 20	Ccr2 21	Cd36 22	Cd44 23	Cdh5 24
Cflar 25	Col3a1 26	Csf1 27	Csf2 28	Csf3 29	Ctgf 30	Cxcl1 31	Hbegf 32
Egr1 33	Eln 34	Eng 35	F7 36	Fabp3 37	Fga 38	Fgb 39	Fgf2 40
Fn1 41	Icam1 42	Icam2 43	Ifnar2 44	Ifng 45	Il10 46	Il13 47	Il1a 48
Il1b 49	Il1r1 50	Il1r2 51	Il1rl1 52	Il2 53	Il3 54	Il4 55	Il5 56
Il6 57	Il7 58	Itga2 59	Itga5 60	Itgax 61	Itgb2 62	Itgb3 63	Itgb5 64
Itgb7 65	Kdr 66	Klf2 67	Lama1 68	Lcat 69	Ldlr 70	Lif 71	Lpl 72
Lypla1 73	Mmp13 74	Mmp1a 75	Mmp3 76	Mmp9 77	Msr1 78	Nfkb1 79	Npy 80
Nr1h3 81	Olr1 82	Pdgfa 83	Pdgfb 84	Pdgfrb 85	Ppara 86	Ppard 87	Pparg 88
Ptgs1 89	Ptgs2 90	Rxra 91	Scarb1 92	Sele 93	Sell 94	Selp 95	Selpl 96
Serpinb2 97	Serpine1 98	Snn 99	Soat2 100	Sod1 101	Sod2 102	Spp1 103	Tgfb1 104
Tgfb2 105	Tgfb3 106	Thbs4 107	Tnc 108	Tnf 109	Tnfaip3 110	Fas 111	Vcam1 112
Vegfa 113	Vwf 114	PUC18 115	Blank 116	Blank 117	AS1R2 118	AS1R1 119	AS1 120
Rps27a 121	B2m 122	Hspcb 123	Hspcb 124	Ppia 125	Ppia 126	BAS2C 127	BAS2C 128

Function of genes in the Oligo GEArray™ Mouse Atherosclerosis Microarray (adapted from http://superarray.com/genetable.php?pcatn=OMM-038)

POSITION	GENEBANK	SYMBOL	DESCRIPTION	FUNCTION
1	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase activity;Mitochondrion;Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity;Glucose metabolism;Glycolysis;Glyceraldehyde-3-phosphate dehydrogenase activity;
2	NM_013454	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	ATP binding;Integral to membrane;Membrane;Integral to plasma membrane;Transport;Cholesterol metabolism;Cholesterol transport;Lipoprotein biosynthesis;
3	NM_009598	Ace	Angiotensin converting enzyme	Hydrolase activity;Integral to membrane;Proteolysis and peptidolysis;Zinc ion binding;Membrane;Extracellular space;Metallopeptidase activity;Carboxypeptidase activity;Peptidyl-dipeptidase A activity;
4	NM_007408	Adfp	Adipose differentiation related protein	Plasma membrane;Nucleus;Protein binding;Membrane;Long-chain fatty acid transport;Lipid particle;Lipid storage;
5	NM_009692	Apoa1	Apolipoprotein A-I	Protein binding;Extracellular space;Lipid transporter activity;Regulation of cholesterol absorption;
6	NM_013474	Apoa2	Apolipoprotein A-II	Extracellular space;Transport;Lipid transporter activity;Lipid transport;Regulation of cholesterol absorption;Lipid binding;Lipoprotein metabolism;Extracellular region;
7	NM_007468	Apoa4	Apolipoprotein A-IV	Extracellular;Extracellular space;Lipid transporter activity;Lipid transport;Regulation of cholesterol absorption;Lipid binding;Lipoprotein metabolism;
8	XM_137955	Apob	Apolipoprotein B	Endoplasmic reticulum;Microsome;Lipid metabolism;Cholesterol transport;Triacylglycerol mobilization;
9	NM_009696	Apoe	Apolipoprotein E	Extracellular;Extracellular space;Heparin binding;Lipid transporter activity;Lipid transport;Lipid binding;Lipoprotein metabolism;
10	NM_007527	Bax	Bcl2-associated X protein	Integral to membrane;Protein binding;Apoptotic mitochondrial changes;Induction of apoptosis;Regulation of apoptosis;Apoptosis;Induction of apoptosis by extracellular signals;Mitochondrial outer membrane;Cytosol;Response to wounding;Induction of apoptosis by intracellular signals;Caspase activation via cytochrome c;Release of cytochrome c from mitochondria;Response to DNA damage stimulus;Protein homooligomerization activity;Insertion of proteins into mitochondrial membranes during the induction of apoptosis;Negative regulation of fibroblast proliferation;Nuclear fragmentation;Regulation of

				caspase activation;
11	NM_009741	Bcl2	B-cell leukemia/lymphoma 2	Integral to membrane;Protein binding;Regulation of apoptosis;Apoptosis;Anti-apoptosis;Mitochondrion;Cytosol;Membrane;
12	NM_009742	Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1d	Regulation of apoptosis;Apoptosis;
13	NM_009743	Bcl2l1	RIKEN cDNA A630035D09 gene	Integral to membrane;Protein binding;Regulation of apoptosis;Apoptosis;Anti-apoptosis;Mitochondrion;Membrane;Response to radiation;Negative regulation of apoptosis;
14	NM_007544	Bid	BH3 interacting domain death agonist	Regulation of apoptosis;Apoptosis;
15	NM_007464	Birc3	Baculoviral IAP repeat-containing 3	Protein binding;Regulation of apoptosis;Apoptosis;Anti-apoptosis;Zinc ion binding;Ubiquitin-protein ligase activity;Protein ubiquitination;Ubiquitin ligase complex;Intracellular;
16	NM_011330	Ccl11	Small chemokine (C-C motif) ligand 11	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
17	NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	Signal transduction;Extracellular;Immune response;Protein binding;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;G-protein-coupled receptor binding;
18	NM_016960	Ccl20	Chemokine (C-C motif) ligand 20	Signal transduction;Extracellular;Immune response;Protein binding;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
19	NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	Signal transduction;Extracellular;Immune response;Inflammatory response;Extracellular space;Chemokine activity;Chemotaxis;Cytokine activity;
20	NM_009912	Ccr1	Chemokine (C-C motif) receptor 1	Integral to membrane;Protein binding;Inflammatory response;G-protein coupled receptor protein signaling pathway;Receptor activity;Rhodopsin-like receptor activity;Immune cell chemotaxis;C-C chemokine receptor activity;G-protein coupled receptor activity;Neuropeptide Y receptor activity;Purinergic nucleotide receptor activity, G-protein coupled;Myeloid blood cell differentiation;
21	NM_009915	Ccr2	Chemokine (C-C motif) receptor 2	Integral to membrane;Immune response;Protein binding;Humoral immune response;Inflammatory response;G-protein coupled receptor protein signaling pathway;Chemotaxis;Defense response;Rhodopsin-like receptor activity;Hemopoiesis;C-C chemokine receptor activity;G-protein coupled receptor activity;Cytokine binding;Cellular defense response (sensu Vertebrata);Perception of pain;Regulation of cell

				migration;
22	NM_007643	Cd36	CD36 antigen	Plasma membrane;Integral to membrane;Cell adhesion;Protein binding;Receptor activity;Membrane;Transport;
23	M27130	Cd44	CD44 antigen	Integral to membrane;Cell adhesion;Protein binding;Receptor activity;Membrane;Hyaluronic acid binding;External side of plasma membrane;Ureteric bud branching;
24	NM_009868	Cdh5	Cadherin 5	Integral to membrane;Calcium ion binding;Cell adhesion;Protein binding;Homophilic cell adhesion;Membrane;Extracellular space;
25	NM_009805	Cflar	CASP8 and FADD-like apoptosis regulator	Proteolysis and peptidolysis;Protein binding;Induction of apoptosis;Regulation of apoptosis;Apoptosis;Caspase activity;
26	NM_009930	Col3a1	Procollagen, type III, alpha 1	Cell adhesion;Cytoplasm;Extracellular matrix structural constituent;Phosphate transport;Collagen;Extracellular matrix structural constituent conferring tensile strength;Extracellular matrix (sensu Metazoa);
27	NM_007778	Csf1	Colony stimulating factor 1 (macrophage)	Integral to membrane;Cell growth and/or maintenance;Extracellular space;Growth factor activity;Cytokine activity;Positive regulation of body size;Positive regulation of odontogenesis (sensu Vertebrata);Regulation of ossification;
28	NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine and chemokine mediated signaling pathway;Cytokine activity;Granulocyte macrophage colony-stimulating factor receptor binding;Dendritic cell differentiation;
29	NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;
30	NM_010217	Ctgf	Connective tissue growth factor	Cell adhesion;Regulation of cell growth;Extracellular;Protein binding;Cell-matrix adhesion;Extracellular space;Heparin binding;Cell migration;Integrin-mediated signaling pathway;Cell differentiation;Angiogenesis;Insulin-like growth factor binding;DNA metabolism;Fibroblast growth factor receptor signaling pathway;Integrin binding;Ossification;Cartilage condensation;Extracellular matrix (sensu Metazoa);
31	NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1	Extracellular;Immune response;Cell growth and/or maintenance;Regulation of cell cycle;Inflammatory response;Intracellular;Extracellular space;Growth factor activity;Chemokine activity;Cytokine activity;
32	NM_010415	Hbegf	Heparin-binding EGF-like growth factor	Integral to membrane;Extracellular space;Epidermal growth factor receptor signaling pathway;Heparin binding;Growth factor activity;Regulation of heart contraction rate;
33	NM_007913	Egr1	Early growth response 1	Nucleic acid binding;DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Transcription factor activity;Zinc

				ion binding;Thymocyte differentiation;
34	NM_007925	Eln	Elastin	Structural molecule activity;Extracellular matrix structural constituent;Myogenesis;Extracellular matrix (sensu Metazoa);Regulation of actin filament polymerization;Stress fiber formation;
35	NM_007932	Eng	Endoglin	Integral to membrane;Cell adhesion;Protein binding;Extracellular space;Angiogenesis;Binding;Heart development;Regulation of transforming growth factor beta receptor signaling pathway;
36	NM_010172	F7	Coagulation factor VII	Hydrolase activity;Calcium ion binding;Proteolysis and peptidolysis;Oxidoreductase activity;Extracellular space;Metabolism;Blood coagulation;Chymotrypsin activity;Trypsin activity;Serine-type endopeptidase activity;Extracellular region;Coagulation factor VIIa activity;
37	NM_010174	Fabp3	Fatty acid binding protein 3, muscle and heart	Transporter activity;Transport;Binding;Lipid binding;
38	NM_010196	Fga	Fibrinogen, alpha polypeptide	Extracellular space;
39	NM_181849	Fgb	Fibrinogen, B beta polypeptide	Extracellular space;Blood coagulation;
40	NM_008006	Fgf2	Fibroblast growth factor 2	Cell proliferation;Signal transduction;Protein binding;Regulation of cell cycle;Extracellular space;Heparin binding;Growth factor activity;Angiogenesis;Positive regulation of cell differentiation;Regulation of retinal programmed cell death;Glial cell differentiation;Fibroblast growth factor receptor binding;
41	NM_010233	Fn1	Fibronectin 1	Cell adhesion;Extracellular;Protein binding;Oxidoreductase activity;Extracellular space;Heparin binding;Acute-phase response;Metabolism;Cell-substrate junction assembly;Wound healing;Extracellular matrix (sensu Metazoa);
42	NM_010493	Icam1	H1 histone family, member 0	Plasma membrane;Integral to membrane;Cell adhesion;Protein binding;Membrane;Cell-cell adhesion;Defense response;
43	NM_010494	Icam2	Intercellular adhesion molecule 2	Integral to membrane;Cell adhesion;Protein binding;Membrane;Cell-cell adhesion;Integral to plasma membrane;Uropod;
44	NM_010509	Ifnar2	Interferon (alpha and beta) receptor 2	Integral to membrane;Cell proliferation;Regulation of transcription from Pol II promoter;Receptor activity;Membrane;Extracellular space;Hematopoietin/interferon-class (D200-domain) cytokine receptor activity;Interferon-alpha/beta receptor activity;
45	NM_008337	Ifng	Interferon gamma	Regulation of cell growth;Extracellular;Immune response;Transcriptional activator activity;Extracellular space;Defense response;Regulation of transcription;Cytokine activity;Interferon-gamma receptor

				binding;Neutrophil chemotaxis;Positive regulation of transcription, DNA-dependent;Positive regulation of MHC class II biosynthesis;Positive regulation of isotype switching to IgG isotypes;Regulation of immune response;Programmed cell death, neutrophils;Positive regulation of interleukin-12 biosynthesis;Positive regulation of interleukin-6 biosynthesis;Positive regulation of chemokine biosynthesis;Positive regulation of interleukin-1 beta secretion;Programmed cell death, inflammatory cells;
46	NM_010548	Il10	Interleukin 10	Extracellular;Immune response;Extracellular space;Cytokine activity;Positive regulation of MHC class II biosynthesis;
47	NM_008355	Il13	Interleukin 13	Extracellular;Immune response;Extracellular space;Cytokine activity;Interleukin-13 receptor binding;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;
48	NM_010554	Il1a	Interleukin 1 alpha	Cell proliferation;Extracellular;Immune response;Regulation of cell cycle;Inflammatory response;Signal transducer activity;Growth factor activity;Interleukin-1 receptor binding;Cytokine and chemokine mediated signaling pathway;Cytokine activity;
49	NM_008361	Il1b	Interleukin 1 beta	Cell proliferation;Extracellular;Immune response;Regulation of cell cycle;Inflammatory response;Signal transducer activity;Growth factor activity;Interleukin-1 receptor binding;Cytokine and chemokine mediated signaling pathway;Cytokine activity;Neutrophil chemotaxis;Positive regulation of interleukin-6 biosynthesis;Positive regulation of chemokine biosynthesis;
50	NM_008362	Il1r1	Interleukin 1 receptor, type I	Integral to membrane;Signal transducer activity;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Transmembrane receptor activity;Interleukin-1, Type I, activating receptor activity;Cytokine and chemokine mediated signaling pathway;Interleukin-1 receptor activity;Interleukin receptor activity;
51	NM_010555	Il1r2	Interleukin 1 receptor, type II	ATP binding;Integral to membrane;Cell surface receptor linked signal transduction;Receptor activity;Membrane;Integral to plasma membrane;Extracellular space;Interleukin-1, Type II, blocking receptor activity;Transport;ATP-binding cassette (ABC) transporter activity;Interleukin-1 receptor activity;Interleukin receptor activity;
52	NM_010743	Il1rl1	Interleukin 1 receptor-like 1	Plasma membrane;Integral to membrane;DNA binding;DNA methylation;Receptor activity;Membrane;Extracellular space;Transmembrane receptor activity;Defense response;Interleukin-1 receptor activity;N-methyltransferase activity;
53	NM_008366	Il2	Interleukin 2	Cell proliferation;Extracellular;Immune response;Inflammatory response;Extracellular

				space;Growth factor activity;Hormone activity;Defense response;Cytokine activity;Cellular defense response;Interleukin-2 receptor binding;
54	NM_010556	Il3	Interleukin 3	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-3 receptor binding;
55	NM_021283	Il4	Interleukin 4	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-4 receptor binding;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;B-cell activation;Negative regulation of osteoclast differentiation;Positive regulation of MHC class II biosynthesis;Positive regulation of isotype switching to IgG isotypes;Regulation of immune response;Regulation of phosphorylation;
56	NM_010558	Il5	Interleukin 5	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Interleukin-5 receptor binding;
57	NM_031168	Il6	Interleukin 6	Extracellular;Immune response;Protein binding;Extracellular space;Acute-phase response;Growth factor activity;Cytokine activity;Interleukin-6 receptor binding;Negative regulation of chemokine biosynthesis;Programmed cell death, neutrophils;
58	NM_008371	Il7	Interleukin 7	Extracellular;Immune response;Growth factor activity;Positive regulation of T-cell differentiation;Cytokine activity;Interleukin-7 receptor binding;Hematopoietin/interferon-class (D200-domain) cytokine receptor binding;Bone resorption;Positive regulation of B-cell proliferation;
59	NM_008396	Itga2	Integrin alpha 2	Integral to membrane;Cell adhesion;Protein binding;Receptor activity;Cell-matrix adhesion;Extracellular space;Integrin-mediated signaling pathway;Integrin complex;Magnesium ion binding;
60	NM_010577	Itga5	Integrin alpha 5 (fibronectin receptor alpha)	Integral to membrane;Cell adhesion;Protein binding;Receptor activity;Cell-matrix adhesion;Extracellular space;Integrin-mediated signaling pathway;Integrin complex;Cell-substrate junction assembly;Synaptosome;Memory;
61	NM_021334	Itgax	Integrin alpha X	Integral to membrane;Cell adhesion;Protein binding;Receptor activity;Cell-matrix adhesion;Integrin-mediated signaling pathway;Integrin complex;Magnesium ion binding;External side of plasma membrane;
62	NM_008404	Itgb2	Integrin beta 2	Integral to membrane;Cell adhesion;Protein binding;Development;Receptor activity;Cell-matrix adhesion;Extracellular space;Integrin-mediated signaling pathway;Integrin complex;Neutrophil chemotaxis;Cellular extravasation;
63	NM_016780	Itgb3	Integrin beta 3	Integral to membrane;Cell adhesion;Protein binding;Oxidoreductase activity;Receptor

				activity;Cell-matrix adhesion;Extracellular space;Metabolism;Integrin-mediated signaling pathway;Integrin complex;Cell-substrate junction assembly;External side of plasma membrane;
64	NM_010580	Itgb5	Integrin beta 5	Integral to membrane;Cell adhesion;Protein binding;Development;Receptor activity;Cell-matrix adhesion;Extracellular space;Integrin-mediated signaling pathway;Integrin complex;
65	NM_013566	Itgb7	Integrin beta 7	Integral to membrane;Cell adhesion;Protein binding;Receptor activity;Cell-matrix adhesion;Extracellular space;Electron transport;Integrin-mediated signaling pathway;Integrin complex;Electron carrier activity;
66	NM_010612	Kdr	Kinase insert domain protein receptor	ATP binding;Transferase activity;Protein amino acid phosphorylation;Integral to membrane;Protein serine/threonine kinase activity;Protein-tyrosine kinase activity;Receptor activity;Transmembrane receptor protein tyrosine kinase activity;Membrane;Protein kinase activity;Extracellular space;Transmembrane receptor protein tyrosine kinase signaling pathway;Kinase activity;Vascular endothelial growth factor receptor activity;Angiogenesis;Cell fate commitment;Endothelial cell differentiation;
67	NM_008452	Klf2	Kruppel-like factor 2 (lung)	Nucleic acid binding;DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Transcription factor activity;Zinc ion binding;Positive regulation of transcription;Transcription regulator activity;
68	NM_008480	Lama1	Laminin, alpha 1	Receptor binding;Structural molecule activity;Cell adhesion;Protein binding;Cell surface receptor linked signal transduction;Extracellular matrix structural constituent;Regulation of cell adhesion;Regulation of cell migration;Morphogenesis of an epithelial sheet;Basal lamina;Basement membrane;Histogenesis;Extracellular matrix (sensu Metazoa);Regulation of embryonic development;Laminin-1;
69	NM_008490	Lcat	Lecithin cholesterol acyltransferase	Transferase activity;Extracellular space;Lipid metabolism;Cholesterol metabolism;Acyltransferase activity;Catalytic activity;Phosphatidylcholine-sterol O-acyltransferase activity;
70	NM_010700	Ldlr	Low density lipoprotein receptor	Integral to membrane;Calcium ion binding;Protein binding;Receptor activity;Membrane;Extracellular space;Endosome;Cholesterol metabolism;Endocytosis;Lipid transporter activity;Lipid transport;Low-density lipoprotein receptor activity;Coated pit;Cholesterol homeostasis;
71	NM_008501	Lif	Leukemia inhibitory factor	Extracellular;Immune response;Extracellular space;Growth factor activity;Cytokine activity;Leukemia inhibitory factor receptor binding;

72	NM_008509	Lpl	Lipoprotein lipase	Hydrolase activity;Extracellular space;Heparin binding;Lipid metabolism;Lipid transporter activity;Catalytic activity;Lipid catabolism;Lipoprotein lipase activity;
73	NM_008866	Lypla1	Lysophospholipase 1	Hydrolase activity;Mitochondrion;Lipid metabolism;Fatty acid metabolism;Catalytic activity;Lysophospholipase activity;
74	NM_008607	Mmp13	Matrix metalloproteinase 13	Hydrolase activity;Calcium ion binding;Proteolysis and peptidolysis;Zinc ion binding;Interstitial collagenase activity;Collagen catabolism;Extracellular space;Metalloendopeptidase activity;Metallopeptidase activity;Extracellular matrix (sensu Metazoa);
75	NM_032006	Mmp1a	Matrix metalloproteinase 1a (interstitial collagenase)	Collagenase activity;
76	NM_010809	Mmp3	Matrix metalloproteinase 3	Hydrolase activity;Calcium ion binding;Extracellular;Proteolysis and peptidolysis;Zinc ion binding;Collagen catabolism;Extracellular space;Metalloendopeptidase activity;Metallopeptidase activity;Stromelysin 1 activity;Extracellular matrix (sensu Metazoa);
77	NM_013599	Mmp9	Matrix metalloproteinase 9	Hydrolase activity;Proteolysis and peptidolysis;Zinc ion binding;Collagen catabolism;Extracellular space;Metalloendopeptidase activity;Metallopeptidase activity;Gelatinase B activity;Extracellular matrix (sensu Metazoa);
78	NM_031195	Msr1	Macrophage scavenger receptor 1	Integral to membrane;Cytoplasm;Receptor activity;Membrane;Scavenger receptor activity;Receptor mediated endocytosis;Endocytosis;Phosphate transport;Lipid transporter activity;
79	NM_008689	Nfkb1	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	Signal transduction;DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Protein binding;Apoptosis;Transcription factor activity;Lymph gland development;Specific transcriptional repressor activity;Negative regulation of interleukin-12 biosynthesis;Negative regulation of transcription, DNA-dependent;
80	NM_023456	Npy	Neuropeptide Y	Extracellular;Extracellular space;Hormone activity;G-protein-coupled receptor binding;Regulation of blood pressure;Neuropeptide signaling pathway;
81	NM_013839	Nr1h3	Nuclear receptor subfamily 1, group H, member 3	DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Electron transporter activity;Transcription factor activity;Receptor activity;Electron transport;Copper ion binding;Steroid hormone receptor activity;Ligand-dependent nuclear receptor activity;Transcription regulator activity;Negative regulation of transcription;

82	NM_138648	Olr1	Oxidized low density lipoprotein (lectin-like) receptor 1	Integral to membrane;Receptor activity;Sugar binding;Low-density lipoprotein receptor activity;Extrinsic to plasma membrane;
83	NM_008808	Pdgfa	Platelet derived growth factor, alpha	Cell proliferation;Cell growth and/or maintenance;Regulation of cell cycle;Membrane;Extracellular space;Organogenesis;Growth factor activity;Regulation of peptidyl-tyrosine phosphorylation;Actin cytoskeleton organization and biogenesis;Cell projection biogenesis;
84	NM_011057	Pdgfb	Platelet derived growth factor, B polypeptide	Cell proliferation;Cell growth and/or maintenance;Regulation of cell cycle;Membrane;Extracellular space;Growth factor activity;Substrate-bound cell migration;Regulation of peptidyl-tyrosine phosphorylation;Actin cytoskeleton organization and biogenesis;Cell projection biogenesis;
85	NM_008809	Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	ATP binding;Transferase activity;Protein amino acid phosphorylation;Integral to membrane;Signal transduction;Protein serine/threonine kinase activity;Protein-tyrosine kinase activity;Signal transducer activity;Receptor activity;Transmembrane receptor protein tyrosine kinase activity;Membrane;Protein kinase activity;Extracellular space;Transmembrane receptor protein tyrosine kinase signaling pathway;Kinase activity;Vascular endothelial growth factor receptor activity;Regulation of peptidyl-tyrosine phosphorylation;
86	NM_011144	Ppara	Peroxisome proliferator activated receptor alpha	DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Transcription factor activity;Receptor activity;Glucose metabolism;Steroid hormone receptor activity;Lipid metabolism;Epidermis development;Ligand-dependent nuclear receptor activity;Fatty acid metabolism;Regulation of fatty acid metabolism;
87	NM_011145	Ppard	Peroxisome proliferator activator receptor delta	Cell proliferation;DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Cell growth and/or maintenance;Transcription factor activity;Negative regulation of transcription from Pol II promoter;Receptor activity;Steroid hormone receptor activity;Lipid metabolism;Epidermis development;Ligand-dependent nuclear receptor activity;Transcriptional repressor activity;Nerve ensheathment;
88	NM_011146	Pparg	Peroxisome proliferator activated receptor gamma	DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Transcription factor activity;Inflammatory response;Cytosol;Transcriptional activator activity;Receptor activity;Regulation of transcription;Steroid hormone receptor activity;Adipocyte differentiation;Ligand-dependent nuclear receptor activity;
89	NM_008969	Ptgs1	Prostaglandin-endoperoxide synthase 1	Cytoplasm;Oxidoreductase activity;Peroxidase activity;Membrane;Extracellular

				space;Microsome;Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen;Prostaglandin-endoperoxide synthase activity;Prostaglandin biosynthesis;Regulation of blood pressure;NOT nucleus;
90	NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2	Cytoplasm;Oxidoreductase activity;Peroxidase activity;Membrane;Extracellular space;Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen;Prostaglandin-endoperoxide synthase activity;Prostaglandin biosynthesis;Regulation of blood pressure;NOT nucleus;
91	NM_011305	Rxra	Retinoid X receptor alpha	DNA binding;Regulation of transcription, DNA-dependent;Nucleus;Transcription factor activity;Receptor activity;Steroid hormone receptor activity;Ligand-dependent nuclear receptor activity;Steroid binding;
92	NM_016741	Scarb1	Scavenger receptor class B, member 1	Integral to membrane;Cell adhesion;Receptor activity;Membrane;
93	NM_011345	Sele	Selectin, endothelial cell	Integral to membrane;Cell adhesion;Protein binding;Membrane;Extracellular space;Sugar binding;Heterophilic cell adhesion;
94	NM_011346	Sell	Selectin, lymphocyte	Integral to membrane;Cell adhesion;Protein binding;Membrane;Extracellular space;Defense response;Sugar binding;Heterophilic cell adhesion;External side of plasma membrane;
95	NM_011347	Selp	Selectin, platelet	Integral to membrane;Cell adhesion;Protein binding;Inflammatory response;Membrane;Extracellular space;Sugar binding;
96	NM_009151	Selpl	Selectin, platelet (p-selectin) ligand	Integral to membrane;Cell adhesion;Protein binding;Extracellular space;Sugar binding;
97	NM_011111	Serpinb2	Serine (or cysteine) proteinase inhibitor, clade B, member 2	Serine-type endopeptidase inhibitor activity;Plasminogen activator activity;
98	NM_008871	Serpine1	Serine (or cysteine) proteinase inhibitor, clade E, member 1	Protein binding;Extracellular space;Serine-type endopeptidase inhibitor activity;Plasminogen activator activity;Regulation of angiogenesis;
99	NM_009223	Snn	Stannin	Integral to membrane;Molecular_function unknown;Biological_process unknown;
100	NM_146064	Soat2	Sterol O-acyltransferase 2	Transferase activity;Integral to membrane;Endoplasmic reticulum;Cholesterol metabolism;Acyltransferase activity;Protein prenyltransferase activity;Protein amino acid prenylation;
101	XM_128337	Sod1	Superoxide dismutase 1, soluble	Cytoplasm;Oxidoreductase activity;Response to oxidative stress;Mitochondrion;Antioxidant activity;Copper, zinc superoxide dismutase activity;Metal ion binding;Superoxide metabolism;Removal of superoxide

				radicals;Activation of MAPK;DNA fragmentation;
102	NM_013671	Sod2	Superoxide dismutase 2, mitochondrial	Oxidoreductase activity;Mitochondrion;Metal ion binding;Superoxide metabolism;Manganese ion binding;Manganese superoxide dismutase activity;Superoxide dismutase activity;
103	NM_009263	Spp1	Secreted phosphoprotein 1	Cell adhesion;Protein binding;Extracellular space;Cytokine activity;Ossification;
104	NM_011577	Tgfb1	Transforming growth factor, beta 1	Protein amino acid phosphorylation;Cell proliferation;Protein binding;Negative regulation of cell proliferation;Regulation of cell cycle;Inflammatory response;Extracellular matrix;Extracellular space;Organogenesis;Growth factor activity;Skeletal development;Defense response;Regulation of cell proliferation;Necrosis;Transforming growth factor beta receptor signaling pathway;Transforming growth factor beta receptor binding;Cell growth;Lymph gland development;Growth;Myogenesis;Regulation of myogenesis;Regulation of protein-nucleus import;
105	NM_009367	Tgfb2	Transforming growth factor, beta 2	Cell proliferation;Regulation of cell cycle;Extracellular space;Growth factor activity;Transforming growth factor beta receptor binding;Cell growth;Growth;Extracellular matrix organization and biogenesis;
106	NM_009368	Tgfb3	Transforming growth factor, beta 3	Cell proliferation;Negative regulation of cell proliferation;Regulation of cell cycle;Extracellular space;Organogenesis;Growth factor activity;Transforming growth factor beta receptor binding;Cell growth;Growth;Embryonic development (sensu Mammalia);Embryonic morphogenesis;
107	NM_011582	Thbs4	Thrombospondin 4	Calcium ion binding;Structural molecule activity;Cell adhesion;Extracellular;Protein binding;Extracellular space;
108	NM_011607	Tnc	Tenascin C	Extracellular space;
109	NM_013693	Tnf	Tumor necrosis factor	Plasma membrane;Cell proliferation;Immune response;Cell growth and/or maintenance;Humoral immune response;Inflammatory response;Positive regulation of cell proliferation;Induction of apoptosis via death domain receptors;Signal transducer activity;Development;Positive regulation of I-kappaB kinase/NF-kappaB cascade;Integral to plasma membrane;Organogenesis;Tumor necrosis factor receptor binding;Defense response;Secretory granule;Regulation of cell proliferation;Cytokine and chemokine mediated signaling pathway;Cellular defense response;Lymph gland development;Cellular extravasation;Positive regulation of osteoclast differentiation;Programmed cell death, transformed cells;Regulation of osteoclast differentiation;
110	NM_009397	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	Hydrolase activity;DNA binding;Nucleus;Protein binding;Cytoplasm;Apoptosis;Zinc ion

				binding;Cysteine-type peptidase activity;
111	NM_007987	Fas	Fas (TNF receptor superfamily member)	Integral to membrane;Signal transduction;Immune response;Apoptosis;Induction of apoptosis via death domain receptors;Receptor activity;Membrane;Transmembrane receptor activity;Programmed cell death, transformed cells;Programmed cell death, inflammatory cells;External side of plasma membrane;Programmed cell death, activated T-cells;
112	NM_011693	Vcam1	Vascular cell adhesion molecule 1	Plasma membrane;Integral to membrane;Cell adhesion;Protein binding;Membrane;Cell-cell adhesion;Extracellular space;
113	NM_009505	Vegfa	Vascular endothelial growth factor A	Cell proliferation;Cell growth and/or maintenance;Anti-apoptosis;Regulation of cell cycle;Mesoderm development;Membrane;Extracellular space;Heparin binding;Growth factor activity;Angiogenesis;
114	NM_011708	Vwf	Von Willebrand factor homolog	Cell adhesion;Protein binding;Extracellular space;Hemostasis;Blood coagulation;Extracellular matrix (sensu Metazoa);
115	L08752	PUC18	PUC18 Plasmid DNA	
116				
117				
118	SA_00005	AS1R2	Artificial Sequence 1 Related 2 (80% identity)(48/60)	
119	SA_00004	AS1R1	Artificial Sequence 1 Related 1 (90% identity)(54/60)	
120	SA_00003	AS1	Artificial Sequence 1	
121	NM_024277	Rps27a	Ribosomal protein S27a	Intracellular;Protein biosynthesis;Ribosome;
122	NM_009735	B2m	Beta-2 microglobulin	Plasma membrane;Integral to plasma membrane;Extracellular space;Defense response;Cellular defense response;Antigen processing, endogenous antigen via MHC class I;MHC class I receptor activity;Antigen presentation, endogenous antigen;
123	NM_008302	Hspcb	Heat shock protein 1, beta	ATP binding;Protein binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;ATP binding;Protein binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;
124	NM_008302	Hspcb	Heat shock protein 1, beta	ATP binding;Protein binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;ATP binding;Protein binding;Mitochondrion;Response to heat;Protein folding;Unfolded protein binding;Response to unfolded protein;

125	NM_008907	Ppia	Peptidylprolyl isomerase A	Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;
126	NM_008907	Ppia	Peptidylprolyl isomerase A	Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;Cytosol;Protein folding;Isomerase activity;Peptidyl-prolyl cis-trans isomerase activity;
127	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	
128	SA_00007	BAS2C	Biotinylated Artificial Sequence 2 Complementary sequence	

APPENDIX VI

Sequence of region upstream of the transcriptional start site for murine IP-10, Mig, I-TAC and CCR2 genes.

IFN-γ-responsiveness for the IP-10 and Mig promoters has been mapped to the sequences shown (Ohmori and Hamilton 1992, Wright and Farber 1991). Putative GAS elements are ISREs are highlighted.

IP-10 promoter (mouse)

[-369]caaatgtaaaaaccatggttagaacctgacttagatatcagctctgtgttttatttatgagagaaggaagatga
gaattaaagccatttccagacttattctgcaaggcactcatctga[-250]tttctcaaa[GAS]cagctcacgcttt
[-228]ggaaagtgaaacc[ISRE]tacctcactcgttaaaaattaaaaggagcacaagaggggagagggaaattccaagt
tcatgggtcacaataaacacaagcaatgccctcggtttacaggggacttccctcgggttgcggagccttgctgagtcatctccaaagt
cagccaatcaggactcagggagggaaactctttgcagataaatactcctcagcagccggcactcgag[-1]aagcgcttc

Mig promoter (mouse)

[-358]Agctttgacttgtgaggaaaggcagtttgagtcgccatatagtgtcatgtcccacgtgggaagtacatctcc
Aaccttcattgtacagcattaatatagtcattgtataatctattccacatccaggtagcaactttgcctggggg
[-212]tggtttcacatccc[ISRE][-198]ttactataaa[GAS]ctccccgtttatgtgaaatggaagtagaacatgca
gaaattccctgggatctgagagtagggttttccccaggacgatcaatttgtggttagtttagttcttctaggtcagctgaggagaccag
ccaatcagagacgggaaggaaaagggatttcctaaataaatatgatccccaagaacatgctctc[-1]taaagacattct

ITAC promoter (mouse)

[-396]gaaattggaaggaaaaaaaaaagtaaagatagatgaaggtcacctggtatgtataatatatgccttatccatatat
gtgtggttatgtgagggaggcaggatgagctgcctggaactgggaccatcttcctgagttggtgggactctgcccagaatccctaca
caatcaaagca[-222]tttctaggaa[GAS]aagccacctgctgagcagctgctgagtgctttcaccttccagtggcttc
ttaactccacagaggggacagtcacagcgctgacttaaggacaaaagagaaactccaggaggcaagaaagctacgtgagtca
aggaggggaattcctgatactgcctgaagattgctggtctataaatgctcctcagactcctcaggttcagccttcctgcct[-1]

CCR2 promoter (mouse)

[-552]Taagcatataggagagtaaaaatcctgagatcagctgatataattcagataaaaatctgaattagaagaattttga
agcaagaataaaaagaaaaaaaggcaaaagttggcaaaagccaaatacagtgccttgaggttttatgaagtttgcagggatat
gtcacagaaatattaataagcagccacgtaggtgtc[-357]tttctagaa[GAS]gctaaaagcaatatttttaagatttatc
tgttttttattttgtgtaagtatctgtgccttaagtcacatatagtttatgtgacttaagtgtgtgcaggtgccaatggagttcaaaagt
gatagatccactaaaactggaattacaggtggttatgagctccctgctgtgggtataggaaatcaacttgggttctatgcaagaaa
agtaagtgcctttgacccctgagccatctctaatcccttcaaaagagttttaaatgggggaaattcatgctcaatgaaaagaatttg
gaatgacattaaggtatttgaacattaaattaacattgcaaccttatcaaaagtg[-1]tgagtgagagcaagggg

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