REGULATION OF TYPE I INTERFERON RESPONSES

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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List of Abbreviations

BMM	Bone marrow macrophage
Bp	Base pairs
CCD	Coiled-coil domain
CDNA	Complementary DNA
CIS	Cytokine inducible SH2 containing protein
CPE	Cytopathic effect
Cpm	Counts per minutes
CSF1	Colony stimulating factor 1
DBD	DNA binding domain
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DRAF	dsRNA activated factor
Dscr1	Down syndrome critical region 1
dsRNA	Double stranded DNA
Epo	Erythropoietin
GAF	Gamma activated factor
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma activated sequence
G-CSF	Granulocyte colony stimulating factor
GH	Growth factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HCL	Hairy cell leukemia
HCV	Hepatitis C virus
Hu	Human
ICSBP	IFN consensus sequence binding protein
IFN	Interferon
IFNAR/Ifnar	Human/murine IFN alpha/beta receptor
IFNGR/Ifngr	Human/murine IFN gamma receptor
Ig	Immunoglobulin

IGF1	Insulin like growth factor 1
IKKε	IκB kinase ε
IL	Interleukin
IPC	IFN producing cell
IRE	IFN response element
IRF	IFN regulatory factor
IRS	Insulin receptor substrate
ISG	IFN stimulated gene
ISGF3	IFN stimulated gene factor 3
ISRE	IFN stimulated response element
IU	International units
JAB	JAK binding protein
JAK	Janus kinase
JH	JAK homology
KDa	Kilodaltons
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MEF	Murine embryo fibroblast
MHCI & II	Major histocompatibility complex class I & II
MRNA	Messenger RNA
MS	Multiple sclerosis
Mu	Murine
NIA	National institute of aging
NK	Natural killer
2'-5' OAS	2'-5' Oligoadenylate synthetase
PBMC	Peripheral blood monocytic cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIAS	Protein inhibitor of activated STAT
PI3K	Phosphatidyl inositol 3' kinase
PKR	Double stranded RNA dependent protein kinase
PRD	Positive regulatory domain

PRL	Prolactin
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
RPMI	Rosewell park memorial institute
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SFV	Semliki forest virus
SH	Src-homology
SHP	SH2 phosphatase
SOCS	Suppressors of cytokine signaling
SPF	Specific pathogen free
ssDNA	Salmon sperm DNA
SSI	STAT induced STAT inhibitor
STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
TBK1	TANK binding kinase 1
TF	Tissue factor
TICAM1	TIR domain containing adaptor molecule 1
TIR	Toll interleukin 1 receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor
TPO	Thrombopoietin
TRIF	TIR regulating IFN factor
TRNA	Transfer RNA
TYK	JAK Tyrosine kinase
Tyr	Tyrosine
UTR	Untranslated region

ADDENDUM

Chapter 1

p 27 line 24: Reference "(Wen *et al.*, 1995)" is missing from reference list. Add to reference list "Wen Z, Zhong Z & Darnell JE Jr. (1995). Maximal activation of transcription by STAT1 and STAT3 requires both tyrosine and serine phosphorylation. *Cell* **82**:241-50."

p 29 line 7: delete "However, studies have demonstrated that STAT1:STAT3 heterodimers can form in response to IFNα (Owczarek *et al.*, 1997)" and read "Although the mice null for each of the STAT proteins are viable with the exception of *Stat3*$^{-/-}$, they do....."

p 33 line 10: add "not the only IFN-inducible cascade" to then read "The JAK/STAT pathway although not the only IFN-inducible cascade, is the best characterised....."

p 34 line 20: Add "in the cytoplasm" to read "Subsequently, this heterodimer binds IRF9 in the cytoplasm to form...." Comment: Still in question as to where IRF9 binds however, data suggest that IRF9 can bind in the cytoplasm and shuttle into the nucleus (Lau *et al.*, 2000). Add to reference list: Lau JF, Parisien J-P & Horvath CM. (2000). Interferon regulatory factor subcellular localization is determined by a bipartite nuclear localization signal in the DNA-binding domain and interaction with cytoplasmic retention factors. *Proc. Natl. Acad. Sci. USA* **97(13)**:7278-83.

Chapter 3

Figures 3.1-3.5: Add to figure legends: "Replicate Northern blots were conducted on same experiments. Northerns shown are representative of replicates.

Figure 3.7: Human Wellferon IFNα was used (mixed alpha preparation).

p 83 line 9: add "hu Wellferon IFNα (Welcome Laboratories, UK), muIFNα4 (Dirk Gewert Biolauncher Ltd), huIFNβ (Serono, Sydney, Australia), muIFNβ (Toray, Japan) huIFNγ (Peprotech Inc., USA) and muIFNγ (Cytolab Ltd., Israel)" at the end of sentence starting "Both human and mouse.....".

Chapter 4

p 93 2nd paragraph: last sentence should read "This result suggested the resistance exhibited by the *Socs1*$^{-/-}$ mice was not significantly different to the survival of uninfected *Socs1*$^{-/-}$ mice."

Figures 4.2, 4.5 and 4.6A: Comment: The mice utilised in figures 4.2 and 4.5 are on a mixed background, while the mice utilised in figure 4.6A are on a bl/6 background. These strain differences along with different virus preparations may account for the difference in wild-type resistance observed in these figures.

p 101 line 33: delete "decreased" add "increased".

Chapter 5

p 114 line 3: add "by PKCδ (protein kinase c δ) in response to type I IFN" to read "....that STAT1 serine phosphorylation by PKCδ (protein kinase C δ) in response to type I IFN may be responsible.....(Uddin *et al.*, 2002)" Add reference to list: Uddin S, Sassano A, Deb DK, Verma A, Majchrzak B, Rahman A, Malik AB, Fish EN & Platanias LC. (2002). Protein kinase C-δ (PKC-δ) is activated by type I interferons and mediates phosphorylation of STAT1 on serine 727. *J. Biol. Chem.* **277(17)**:14408-16.

Chapter 6

Figure 6.1: add references into figure legends, Figure 6.1A "(Hwang *et al.*, 1995)", Figure 6.1B "(Hertzog *et al.*, unpublished)".

Chapter 7

p 129 line 8: add "the concept" to read "....search for changes in gene expression to confirm the concept that some chain-specific signals are transduced in the *Ifnar*$^{-/-}$ mice."

The experiments discussed throughout this chapter were part of a pilot experiment to determine if the two *Ifnar*$^{-/-}$ mice models could transduce signals independently in response to type I IFN treatment. The presence of a signal in each bone marrow macrophages from each of the single *Ifnar*$^{-/-}$ mice was confirmed by real time PCR using different RNA samples from array experiments.

Abstract

The type I interferons (IFNs) are an important group of cytokines in host defence, playing important roles in the regulation of physiological and pathological processes. They elicit antiviral, antiproliferative, immunoregulatory and antitumor effects in a variety of cell types via receptor components IFNAR1 and IFNAR2 and the JAK/STAT pathway. While JAK/STAT signaling can be regulated by the Suppressors of Cytokine Signaling (SOCS) protein family, the specific effects on IFN signaling were unknown at the outset of this work.

In vitro studies have demonstrated both type I and II IFNs induce *Socs1* and *Socs3* expression. Once induced, the SOCS1 and SOCS3 proteins can suppress both type I and II IFN actions, SOCS1 more so than SOCS3. The importance of SOCS1 *in vivo* was facilitated by the generation of the *Socs1*$^{-/-}$ mice. These mice suffer from pathologies resulting in neonatal death from hypersensitivity to IFNγ. However the role of SOCS1 in the type I IFN signaling system was unknown. In this thesis, genetic crosses of *Socs1*, *Ifnar1* and *Ifnar2* gene targeted mice have been used to investigate molecular signaling mechanisms and susceptibility to infections to demonstrate a role for SOCS1 in mediating type I IFN responses *in vivo*.

Socs1$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice display enhanced type I IFN antiviral responses to *S*emliki *F*orest *v*irus (SFV). This was demonstrated by increased survival and lower viral titres in organs after infection. With the use of a neutralising anti-IFNα/β antibody, the increased resistance to SFV was demonstrated to result from type I rather than type II IFN. The molecular mechanisms were examined to elucidate how this increased resistance to infection was occurring. *In vitro* studies using *Socs1*$^{+/+}$, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ murine embryo fibroblasts demonstrated by measuring 2'-5' oligoadenylate synthetase, an IFN regulated enzyme, that cells lacking *Socs1* displayed a sustained signal rather than an increased magnitude of the response.

Crossing *Ifnar1* and *Socs1* targeted mice demonstrated that *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice survive past weaning unlike the *Socs1*$^{-/-}$ however died by 14 weeks of age.

Interestingly, the pathology that *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice suffer was different to that of *Socs1*$^{-/-}$ mice, instead resembling the pathology of *Socs1*$^{-/-}$ *Ifng*$^{+/-}$. There was infiltration of T cells in the cornea (often resulting in the formation of ulcers), skeletal muscle and the lungs resulting in thickening of the alveolar wall. The *Ifnar2* crosses to the *Socs1*$^{-/-}$ highlighted a difference between the two *Ifnar*$^{-/-}$ mice, as these mice die during the neonatal period similar to the *Socs1*$^{-/-}$.

Differences between the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice were examined further using microarray analysis of IFN-regulated genes. This analysis revealed the first molecular evidence that each of the two *Ifnar*$^{-/-}$ mice were capable of initiating signaling. Thus, the two components of the type I IFN receptor complex can and do act independently of each other.

These studies on the mechanisms for regulating the nature and duration of IFN signaling will provide an understanding of both the inhibition and activation of signaling pathways in order to understand the beneficial and harmful effects of cytokines and ultimately to further capitalise on their therapeutic potential.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Jennifer Eve Fenner

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List of Conference Presentations

Part of the work in this thesis was presented as oral presentations at the following conferences:

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Fenner JE, Starr R, Cornish A, Alexander WS, Hilton DJ & Hertzog PJ. (2002). SOCS1 Regulation of Type I Interferon Responses. Presented at: *The Monash Institute of Reproduction and Development Postgraduate Symposium*, Melbourne, Victoria, Australia.

Fenner JE, Starr R, Cornish A, Alexander WS, Hilton DJ & Hertzog PJ. (2001). Regulation of Type I IFN Responses by SOCS Proteins. Presented at: *The 8th Conference of the Immunology Group of Victoria*, Mt Buffalo, Victoria, Australia.

Fenner JE, Starr R, Alexander WS, Hilton DJ & Hertzog PJ. (2001). Regulation of Type I IFN Responses by SOCS Proteins. Presented at: *The Monash Institute of Reproduction and Development Postgraduate Symposium*, Melbourne, Victoria, Australia.

Fenner JE, Starr R, Alexander WS, Hilton DJ & Hertzog PJ. (2000). Signaling by Type I Interferons and SOCS Regulation of Type I Interferon Responses. Presented at: *The Monash Institute of Reproduction and Development Postgraduate Symposium*, Melbourne, Victoria, Australia.

Part of the work in this thesis was presented as posters at the following conferences:

Fenner JE, Starr R, Cornish A, Alexander WS, Hilton DJ & Hertzog PJ. (2002). Regulation of Type I IFN Responses by SOCS Proteins. Presented at: *The Annual Meeting of the International Society for Interferon and Cytokine* Research, Turin, Italy.

Fenner JE, Starr R, Owczarek C, Scott B, Alexander WS, Hilton DJ & Hertzog PJ. (2001). Regulation of Type I IFN Responses by SOCS Proteins. Presented at: *The 13th Lorne Cancer Conference*, Lorne, Victoria, Australia.

Fenner JE, Owczarek C, Alexander WS, Starr R, Hilton DJ, Kola I & Hertzog PJ. (2000). SOCS Gene Expression Negatively Regulates Interferon Responses. Presented at: *The 21st Annual Conference on the Organisation and Expression of the Genome*, Lorne, Victoria, Australia.

CHAPTER 1

Literature Review

1.1 INTRODUCTION

Interferons (IFNs) are a family of cytokines. This family was discovered through experiments that detected a soluble factor (IFN) produced in response to inactivated viruses and inhibited viral replication (Issac & Lindemann, 1957; Stites *et al.*, 1997). Subsequently, IFNs have also been shown to exhibit potent antiproliferative, antitumor and immunomodulatory properties (Pestka *et al.*, 1987) (Figure 1.1). As a result of these pleiotrophic effects, IFNs are believed to play important roles in the regulation of physiological and pathological processes.

There are two types of IFN, type I and type II. The type I IFNs consist of 13 α (human and murine) subtypes, single β, δ, κ, τ and ω subtypes and limitin (Todokoro *et al.*, 1984; Weissmann & Weber, 1986; De Maeyer & De Maeyer-Guignard, 1988; Lefèvre *et al.*, 1998; Roberts *et al.*, 1999; Oritani *et al.*, 2001; Hardy *et al.*, 2002; Nardelli *et al.*, 2002; Oritani *et al.*, 2003). In contrast, the type II IFN group consists of a sole member, IFNγ (Young *et al.*, 1997). In order to elicit their biological effects, the IFNs bind to type-specific receptors. The type I IFNs bind to the type I *IFNα r*eceptor (IFNAR) that consists of two chains, IFNAR1 and IFNAR2 (Pestka *et al.*, 1987; Hertzog *et al.*, 1994). IFNγ, the type II IFN, binds to the *IFNγ r*eceptor (IFNGR) that consists of a dimer of the two chains IFNGR1 and IFNGR2 (Schindler & Darnell, 1995; Bach *et al.*, 1997; Stark *et al.*, 1998). Both types of IFN have been shown to signal through *J*anus *k*inases (JAKs) and *s*ignal *t*ransducers and *a*ctivators of *t*ranscription (STAT). There has been considerable progress in the characterisation of IFN signaling pathways. In addition, it is clear that a system of negative regulation exists through the actions of soluble receptors, negative signaling factors including tyrosine phosphatases, *p*rotein *i*nhibitors of *a*ctivated *S*TAT (PIAS) and *s*uppressors *o*f *c*ytokine *s*ignaling (SOCS) proteins and transcriptional repressors such as *i*nterferon *r*egul*a*tory *f*actor (IRF) 2 and *I*FN *c*onsensus *s*equence *b*inding *p*rotein (ICSBP) (Kim & Maniatis, 1996; Strous *et al.*, 1996; Chung *et al.*, 1997; Endo *et al.*, 1997; Naka *et al.*, 1997; Neel, 1997; Starr *et al.*,

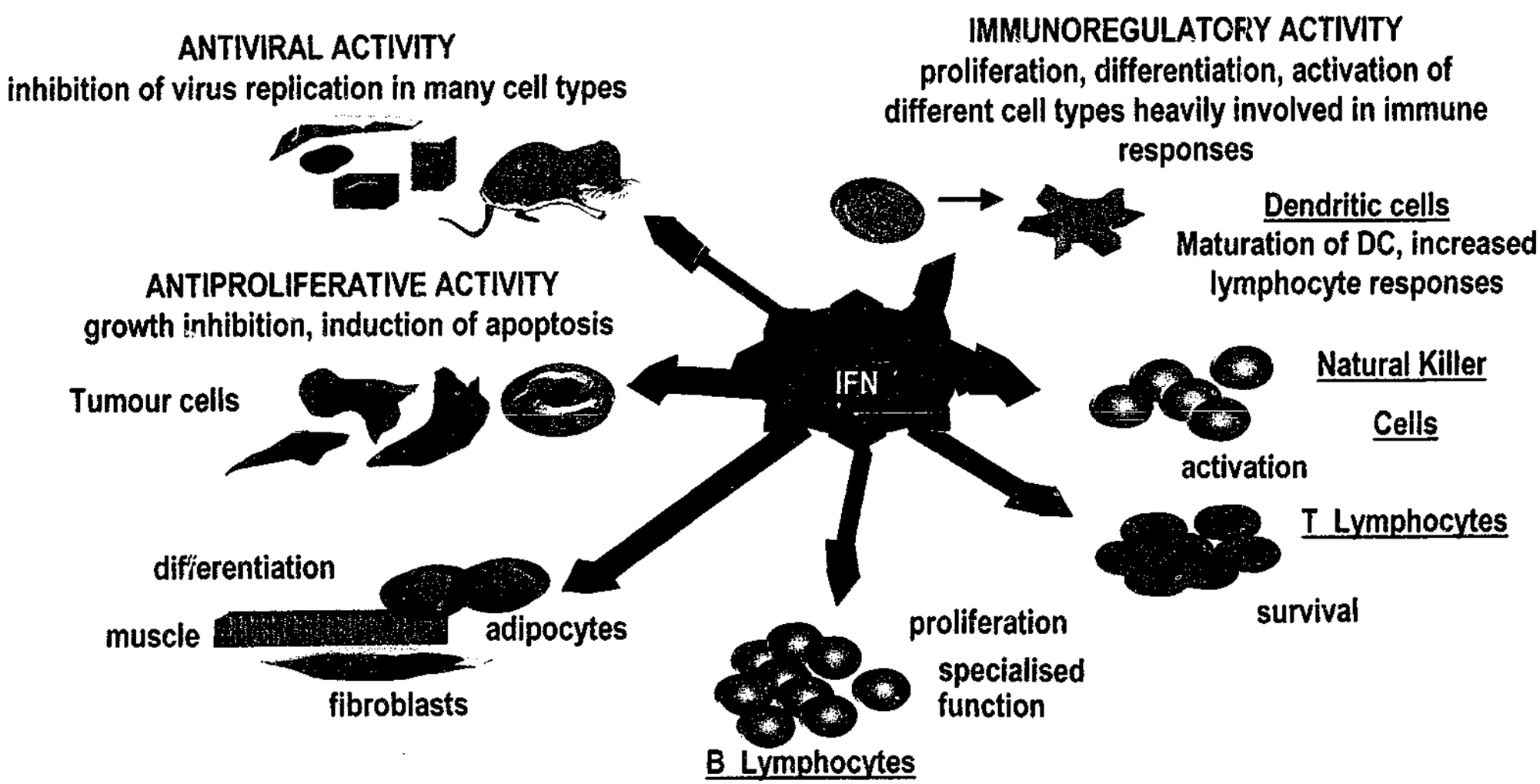


Figure 1.1: Biological Actions of IFN.

IFNs are pleiotrophic cytokines that can effect numerous cells within the body, ranging from very specialised to broad functions (Issac & Lindemann, 1957; Pestka *et al.*, 1987; Stites *et al.*, 1997).

1997; Hardy *et al.*, 2001; Irie-Sasaki *et al.*, 2001). While numerous components that influence IFN signaling have been identified, there is still much to be elucidated on how these components function together to regulate specific effects of particular IFNs *in vivo*.

The pleiotrophic biological effects elicited by the IFN family have made them candidates for the development of effective therapeutic agents. In fact, the IFNs are currently the most widely used cytokines in clinical use. IFNs are most commonly used in the treatment of diseases such as hepatitis B and C, multiple sclerosis, genital warts and some forms of cancer such as Kaposi's sarcoma, hairy cell leukaemia, malignant melanoma and chronic myelogenous leukaemia (Davis, 1999; DeMaeyer & DeMaeyer-Guignard, 1988; Pitha, 2000). However, with the beneficial effects of IFN based therapies come side effects that arise from the prolonged treatment regimes required for chronic diseases such as cancer, chronic infections and autoimmune diseases. These side effects include nausea, fever, headaches, toxicity and in extreme cases the development of autoimmune diseases (Gutterman *et al.*, 1994). Furthermore, the toxicity arising from IFN treatment limits the doses that can be administered. Therefore, a greater understanding of how both positive and negative IFN signals are regulated will enable a better understanding of disease processes involving IFNs, as well as lead to more selective IFN therapies with an increased efficacy and specificity with decreased side effects. The research outlined in this thesis addresses the regulation of IFN signaling by SOCS1, and thus this review focuses on the current understanding of the biological functions and regulation of signaling in the IFN system.

1.2 INTERFERONS

1.2.1 Type I Interferons

The type I IFNs are silent workers of the immune system. They are the first line of defence in a viral infection, acting as part of the innate immune response,

and are subsequently able to regulate further adaptive immune mechanisms to clear foreign pathogens. Thus the type I IFNs serve as a crucial link between the innate and adaptive immune response (Le Bon & Tough, 2002).

Currently there are at least 13 α subtypes, single β, δ, κ, multiple τ, ω and limitin which are classified into the group of type I IFNs. The 13 α and single β subtypes have been described in both human and mouse (Hardy *et al.*, 2003). In contrast, the IFNδ gene has only been found in pigs, the IFNτ gene has been described in cattle, sheep and more recently a related gene has been annotated in mouse and human (Hardy *et al.*, 2003). IFNω has been identified in both cattle and humans (Roberts *et al.*, 1998). Interestingly, bovine and ovine IFNτ unlike the other type I IFNs, is only very weakly induced if at all by virus. However its expression is temporal and restricted to concepti of ruminants immediately prior to implantation by embryonic trophectoderm (Roberts *et al.*, 1999; Rosenfeld *et al.*, 2002). Clearly the type I IFNs encompass a large family of proteins that have been identified in numerous species, highlighting their importance.

The IFNα subtypes share a 75% to 99% amino acid identity, while the sole IFNβ and the more recently identified IFNκ are the most divergent of all type I IFNs sharing only 30% amino acid identity to a consensus IFNα sequence (Nardelli *et al.*, 2002; Weissmann & Weber, 1986). There is a 62% amino acid identity between the consensus IFNα sequence and the IFNω (De Maeyer & De Maeyer-Guignard, 1988). The type I IFNs are encoded by intronless genes that are approximately 800 base pairs in length (Henco *et al.*, 1985) and are clustered onto human chromosome 9 (Diaz *et al.*, 1994) and mouse chromosome 4 (Kelley & Pitha, 1985). Historically, leukocytes were described as the predominant IFNα protein producers, while IFNβ production was ascribed primarily to fibroblasts. The current understanding is that the majority of cell types in the body are capable of producing all type I IFNs (Le Bon *et al.*, 2001). However, there are a group of cell types termed *i*nterferon *p*roducing *c*ells (IPCs) or plasmacytoid cells that are capable of producing 1000-fold higher levels of type I IFNs than other cell types in response to viral infection (Colonna

et al., 2002; Fitzgerald-Bocarsly, 1993). These cells have been described as a unique haematopoietic cell type, thought to be of lymphoid origin (Siegal *et al.*, 1999; Liu, 2001). Subsequently these IPCs were further classified as a subset of dendritic cells (Le Bon & Tough, 2002) (Table 1.1). Therefore, it can be said that while most cells in the body are capable of producing type I IFNs in response to various stimuli, there are specialised cells (IPCs) that can generate large amounts when required.

The three-dimensional structures of the type I IFNs place them as members of a family of helical cytokines (Sprang & Bazan, 1993). Analysis of the three dimensional structure of the IFN proteins has revealed a high degree of similarity in overall structure (Runkel *et al.*, 1998a). All the IFNα proteins possess four conserved cysteine residues that form two intramolecular disulphide bonds. Studies investigating the crystallography together with mutagenesis of the type I IFN proteins have revealed particular domains important for biological activity of the protein and in receptor interactions (Mitsui *et al.*, 1993; Uze *et al.*, 1994; Sen, 2001). After analysis of the IFNβ tertiary structure it was concluded that this divergent IFN was comprised of five α helices (Senda *et al.*, 1992). Mutational studies of the IFNβ protein have revealed that there are different domains from the IFNα proteins that are important in IFNβ functions (Runkel *et al.*, 1998b).

The type I IFN genes are predominantly induced by viral infection, with the exception of IFNτ. The mechanisms involved in the induction of the type I IFN gene expression and hence production of proteins, have been mostly characterised in response to viral infection. However, they are also induced by non-viral molecules such as *l*ipo*p*oly*s*accharide (LPS) via *t*oll *l*ike *r*eceptor (TLR) 4, poly I:C via TLR3, purine analogues such as imiquimod via TLR7, CpG DNA via TLR9, M-CSF, IL4, TNFα and IFNγ (Hamilton *et al.*, 1996; Kirchner *et al.*, 1986b; Sampson *et al.*, 1991; Vogel *et al.*, 1982; Zhou *et al.*, 1995). It is noteworthy that there are substantial differences in the promoter sequences of individual type I IFN genes, which might explain their differential expression, which will be discussed later.

Table 1.1: Interferons and their Properties.

(Todokoro *et al.*, 1984; Weissmann & Weber, 1986; De Maeyer & De Maeyer-Guignard, 1988; Lefèvre *et al.*, 1998; Roberts *et al.*, 1999; Oritani *et al.*, 2001; Hardy *et al.*, 2002; Nardelli *et al.*, 2002; Oritani *et al.*, 2003)

	IFNα	IFNβ	IFNδ	IFNκ	IFNω	IFNτ	Limitin	IFNγ
IFN type	Type I	Type I	Type I	Type I	Type I	Type I	Type I	Type II
Receptor	IFNAR	IFNAR	IFNAR	IFNAR	IFNAR	IFNAR	IFNAR	IFNGR
Chromosome Location (hu/mu)	9 (human) 4 (mouse)	9 (human) 4 (mouse)	-	9 (human)	9 (human)	9 (human)	4 (mouse)	12 (human) 10 (mouse)
Number of genes (species)	13 (human) 13 (mouse)	1 (human) 1 (mouse)	1 (porcine)	1 (human)	6 (human)	4 (ruminants)	1 (mouse)	1 (human) 1 (mouse)
Molecular weight	15-21 kDa (hu/mu)	22 kDa (hu/mu)	19 kDa (porcine)	25 kDa (hu) 23 kDa (mu)	24 kDa (hu)	22 kDa	20 kDa (mu)	19 kDa (hu) 18 kDa (mu)
Amino Acid Identity (to IFNα Consensus Sequence)	80-99%	30%	42%	30%	60-70%	50%	30%	10%
Major producing cell types	Macrophages, NK cells, Dendritic cells (IPCs)	Macrophages, NK cells, Dendritic cells (IPCs)	Trophecto-derm	Epidermal keratinocytes	Lymphocytes	Lymphocytes	T Lymphocytes in Spleen & Thymus	NK cells, NK T cells, CD8 and CD4 cells
Major inducer	Virus	Virus, LPS	Pregnancy	Virus	Virus	Pregnancy	Virus	Mitogen
Antiproliferative activity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Antiviral activity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Immunoregulatory	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Antitumor activity	Yes	Yes	-	-	-	-	-	Yes

1.2.1.1 Autocrine Production of Type I Interferon

The majority of cell types within the body are capable of producing IFN, which can then act in an autocrine or paracrine manner in order to fight infection and also induce ongoing production of IFN (Hertzog *et al.*, 1994). In various organs of mice, transcripts of the type I IFNs could be amplified by RT-PCR (Brandt *et al.*, 1993). Immunohistochemical studies using monoclonal, polyclonal or antipeptide antibodies have been able to identify IFN production in numerous organs of normal individuals (Jilbert *et al.*, 1986; Khan *et al.*, 1989; Greenway *et al.*, 1992; Hwang *et al.*, 1994). These observations imply that there is a constitutively low level of IFN production occurring even in the absence of viral infection (Hertzog *et al.*, 1994). In normal cells this low level of autocrine IFNα/β production enables these cells to be primed to increase their production of further IFN after viral infection (Taniguchi & Takaoka, 2001). Low levels of type I IFN production/signaling also prepare cells to react more efficiently to IFNγ (Mitani *et al.*, 2001; Takaoka *et al.*, 2000).

1.2.1.2 Type I IFN Induction by IRF Family Members

The IRF family of transcription factors are involved in the production of IFNα/β by binding to *cis*-acting elements located within the promoter region of *Ifna/b* genes (Fujii *et al.*, 1989; Tanaka *et al.*, 1993). Firstly, IRF1 was demonstrated to activate IFNα/β promoters by binding to *p*ositive *r*egulatory *d*omain *I* (PRDI) elements in the promoter regions of *Ifna/b* genes, through a series of cDNA transfection experiments. High IRF1 levels induced endogenous *Ifna/b* genes in various cell lines (Harada *et al.*, 1990; Fujita *et al.*, 1989). IRF2 on the other hand appeared to repress the transcriptional activation of IRF1, by binding to PRDI elements blocking and subsequently inhibiting IRF1 binding. This led to the concept that IRF1 and IRF2 worked together as a transcriptional activator and suppressor, respectively, of *Ifna/b* genes. However, the generation of mice lacking *Irf1* altered the hypothesis that *Irf1* was required for the production of type I IFNs as these mice were still able to produce normal levels of type I IFNs in response to virus (Ruffner *et al.*, 1993; Matsuyama *et al.*, 1993; Reis *et al.*, 1994).

Viral infection induces the production of IFNα4/β predominantly by IRF3 transactivation, while the remaining IFNα subtypes are produced by the actions of IRF7 (Nguyen *et al.*, 1997; Marie *et al.*, 1998; Mamane *et al.*, 1999; Taniguchi *et al.*, 2001). Prior to viral infection, IRF3 is present in an inactive form in the cytoplasm (Lin *et al.*, 1998). After infection, *T*ANK-*b*inding *k*inase *1* (TBK1) and possibly *I*κB *k*inase-ε (IKKε) can activate IRF3 by phosphorylating its C-terminal serine residues (Juang *et al.*, 1998; Lin *et al.*, 1998; Sato *et al.*, 1998; Wathelet *et al.*, 1998; Yoneyama *et al.*, 1998; Weaver *et al.*, 1998; Fitzgerald *et al.*, 2003). Phosphorylated IRF3 subsequently translocates to the nucleus and associates with its co-activators, CBP/p300 also known as *ds*RNA activated factor (DRAF) (Daly & Reich, 1993). Together they form a transcriptional unit that binds to IRF binding elements within the IFNβ promoter (Whittemore & Maniatis, 1990; Lin *et al.*, 1998; Sato *et al.*, 1998; Wathelet *et al.*, 1998; Yoneyamam *et al.*, 1998). In subsequent studies, IFNα4 has been demonstrated to be the only IFNα to be induced in *Stat1*$^{-/-}$ mice, suggesting that it is unique from the remaining IFNαs (Marie *et al.*, 1998). It was demonstrated to be induced by IRF3 in a similar fashion to IFNβ, with the promoter of IFNα4 containing sequences similar to PRDI (Au *et al.*, 1993; Braganca *et al.*, 1997; Genin *et al.*, 1995; Marie *et al.*, 1998; Näf *et al.*, 1991).

IFNα4 and IFNβ gene induction is induced by IRF3 and requires no new protein synthesis (Marie *et al.*, 1998), therefore these IFN subtypes have been described as immediate early type I IFN genes. The remaining IFNα subtypes, in particular IFNα2, α5, α6 and α8 require new protein synthesis for expression and thus exhibit a delayed induction (Marie *et al.*, 1998). This occurs by IFNα4 and β acting via the type I IFN receptors to activate the JAK/STAT pathway inducing the formation of ISGF3 to translocate to the nucleus to induce not only antiviral ISGs, but also IRF7. Once induced, IRF7 is phosphorylated by TBK1 and possibly IKKε kinases in virus infected cells (Sharma *et al.*, 2003). Subsequently, in order for the delayed IFNα genes to be transcriptionaly activated, IRF7 translocates into the nucleus where it binds to PRDI and III like sequences within the promoter regions of the IFNα genes other than IFNα4. (Marie *et al.*, 1998). Experiments using *Ifnar*$^{-/-}$ or *Stat1*$^{-/-}$ cells have

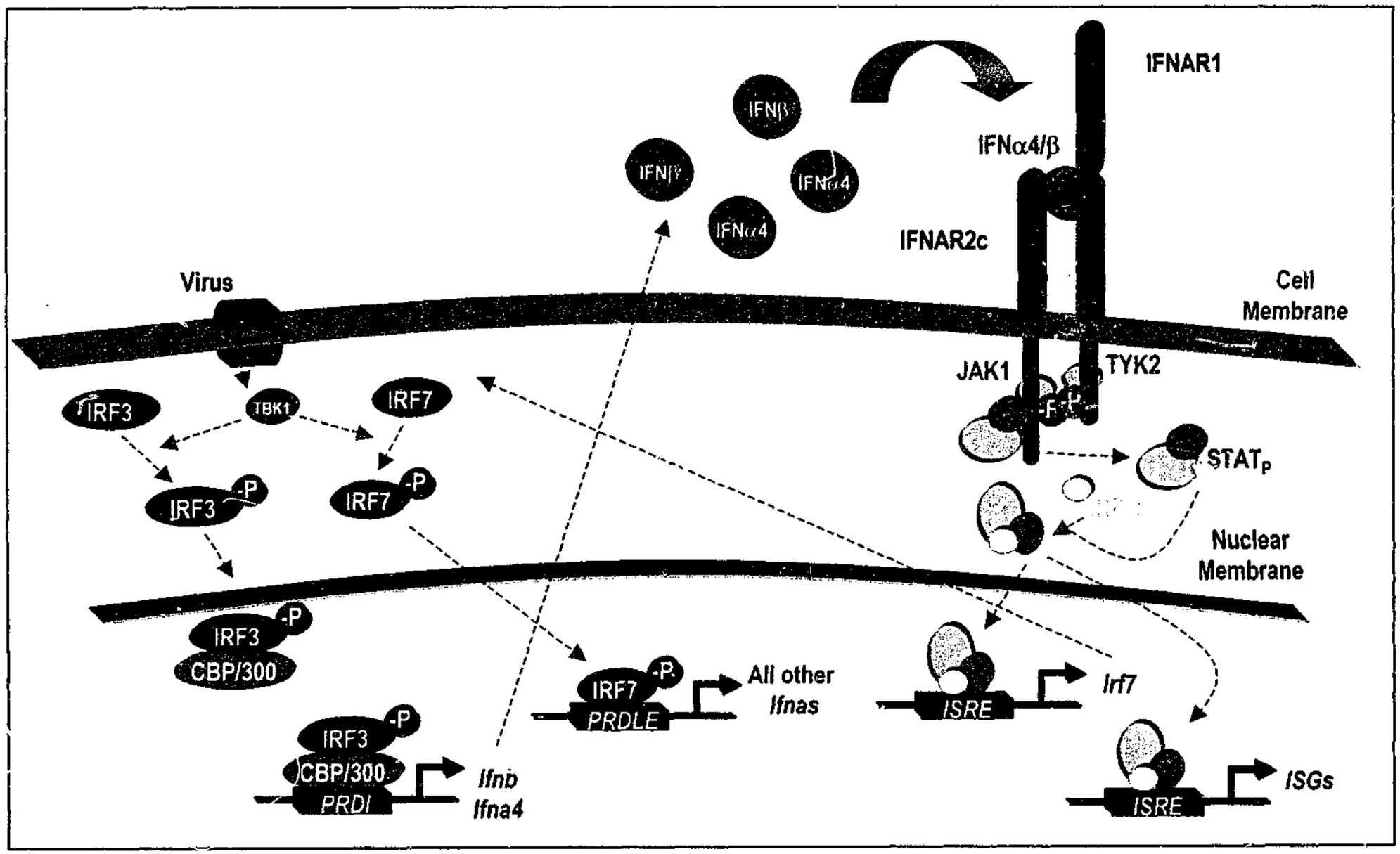
demonstrated that IFNβ and IFNα4 production is independent of these factors. In contrast, both the type I IFN receptor and STAT1 is required for the production of the delayed IFNα subtypes to occur in fibroblasts in response to Newcastle disease virus (Durbin *et al.*, 1996; Marie *et al.*, 1998) (Figure 1.2). In the absence of the *Ifnb* gene, all type I IFN production appears to be reduced in response to virus (Deonarain *et al.*, 2000) illustrating that the mechanism of induction of the delayed genes is dependent on immediate early genes. This suggests that IFNα4/β plays an irreplaceable role in priming cells for the induction of the late onset *Ifna* genes (Erlandsson *et al.*, 1998; Marie *et al.*, 1998).

1.2.1.3 Activation of Type I IFN Gene Expression via Toll Like Receptors (TLRs)

The TLRs have an important role within the innate immune system of responding to pathogens (Medzhitov, 2001). They function as a microbial surveillance system, which once activated by invading pathogens, initiates signaling pathways to eradicate the invading microorganisms. Ten TLR members have been identified recently (Chuang & Ulevitch, 2001; Aderem & Ulevitch, 2000; Akira *et al.*, 2001; O'Neill & Dinarello, 2000; Rock *et al.*, 1998) with TLR3, 4, 7 and 9 having been implicated in the production of IFNβ (Toshchakov *et al.*, 2002). More specifically, TLR3 recognises dsRNA in the form of poly I:C, that subsequently activates IRF3 by IKKε and TBK1 kinases. These kinases are activated through a novel adapter molecule, *t*oll *i*nterleukin 1 *r*eceptor (*TIR*) domain containing *a*dapter *m*olecule *1* (TICAM1) also known as *TIR* regulating *IFN* factor (TRIF) (Alexopoulou *et al.*, 2001; Oshiumi *et al.*, 2003; Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). Likewise, TLR4 recognises LPS which in turn activates IRF3 via TRIF, which is able to initiate IFNβ production as described above by binding to specific elements within the promoter region of the *Ifnb* gene (Poltorak *et al.*, 1998). TLR7 signaling has been described as MyD88 dependent and currently has no known natural ligand, however it has been demonstrated to be activated by synthetic compounds that have antiviral activity such as imidazoquinoline compounds, imiquimod and its derivative

Figure 1.2: Type I IFN Production by Virus Infection.

Virus infection results in TBK1-mediated phosphorylation of IRF3 which then translocates to the nucleus where it binds to CBP/300 to form DRAF which can bind to the promoter regions of the *Ifnb* and *Ifna4* genes. Subsequently IFNα4/β is secreted from the cell and able to bind to the IFNAR on the cell surface. This activates the JAK/STAT pathway which results in the induction of IRF7. IRF7 is then phosphorylated by TBK1 in response to virus and subsequently translocates to the nucleus where it binds to the promoter regions of all other *Ifna* genes inducing their expression.


IFNAR1
IFNβ
IFNα4/β
IFNγ
IFNα4
IFNα4
IFNAR2c
Virus
Cell
Membrane
JAK1
TYK2
P
P
TBK1
IRF3
IRF7
IRF3
-P
IRF7
-P
STAT$_P$
Nuclear
Membrane
IRF3
-P
CBP/300
IRF7
-P
PRDLE
All other
Ifnas
ISRE
Irf7
IRF3
-P
CBP/300
PRDI
Ifnb
Ifna4
ISRE
ISGs

resiquimod (R-848) (Hemmi *et al.*, 2002). TLR 9 recognises unmethylated CpG motifs in bacteria or viruses and functions by stimulating B cell proliferation and activation of macrophages and dendritic cells (Akira & Hemmi, 2003). Thus the type I IFNs are capable of being induced by numerous members of the TLR family.

1.2.2 Type II Interferon

The sole type II IFN, IFNγ has no structural similarity to the type I IFNs however it does share some functional similarity, particularly the antiviral response (Sen, 2001). The *Ifng* gene is located on chromosome 12 in humans and chromosome 10 in the mouse and contains three introns (Pestka *et al.*, 1987). IFNγ has classically been described as the immune IFN, and has a substantial role in the regulation of the adaptive immune response, more specifically to promote Th1 like responses (Farrar & Schreiber, 1993). However, IFNγ is able to elicit many of the same effects as the type I IFNs including regulation of cell and tumour growth, antiviral activity and immunomodulatory functions. This cytokine is able to enhance antigen presenting cell function by stimulating phagocytosis and up regulating the MHCI and II complexes (Boehm *et al.*, 1997). IFNγ also plays a role in the IgG heavy chain class switching and stimulating the production of cytokines which play important immunomodulatory roles such as *t*umour *n*ecrosis *f*actor α (TNFα) and IL12 (Boehm *et al.*, 1997; Stark *et al.*, 1998). Thus by performing these functions as well as being able to induce antiviral genes, IFNγ has the ability to prime cells for defence against foreign molecules.

The generation of *Ifng*$^{-/-}$ mice has demonstrated that IFNγ is not essential for the development of the immune system under *s*pecific *p*athogen *f*ree (SPF) conditions (Dalton *et al.*, 1993). However it was demonstrated that IFNγ was required to prime macrophages for nitric oxide production following viral infection and thus initiate pathways to combat the infection as well as to enhance the expression of MHCII molecules during infection (Dalton *et al.*, 1993). *Ifng*$^{-/-}$ mice were also unable to combat a sublethal dose of the bacterial

pathogen *Mycobacterium bovis*. This increases the expression of MHCII on macrophages in order to combat the bacterial infection. Splenocytes, lymphocytes and NK cells all demonstrated less antimicrobial activity than in the wild-type controls (Dalton *et al.*, 1993). These studies illustrate the importance of IFNγ to a fully functional immune system.

1.2.2.1 Type II Interferon Production

In contrast to the type I IFNs, IFNγ production is induced by mitogenic or antigenic stimuli and can only be produced by particular immune cells in the body. These include CD4 T helper cells, CD8 cytotoxic cells and *n*atural *k*iller (NK) cells and *n*atural *k*iller *T* (NKT) cells (Godfrey *et al.*, 2000; Young *et al.*, 1997; Donnelly *et al.*, 1995). IFNγ production is largely cytokine initiated mainly by IL12 production that is induced by the presence of either bacteria, protozoa or viruses in monocytes and macrophages. Once IL12 has been produced it then acts on NK cells, CD4 and CD8 T cells to promote the production of IFNγ. It is believed that the presence of regulatory elements within the promoter of the *Ifng* gene give rise to cell specific expression (Sen, 2001). Other cytokines and factors such as IL1, IL2, growth factors, estrogen and IFNα/β will also increase the production of IFNγ (Sen, 2001).

1.3 INTERFERONS AND DISEASE

IFNs are important in host defence against infectious diseases, cancers, immune disorders and inflammation. Historically, type I IFNs have been involved in antiviral immune responses while IFNγ has been thought of as an immune response cytokine involving T cell activity (Pestka *et al.*, 1987; Müller *et al.*, 1994; Hwang *et al.*, 1995). However, as the knowledge of IFN signaling increases, the overlap between type I and II IFNs functions have become clearer.

The pleiotrophic biological effects of the type I IFNs make them potential therapeutic agents. To be able to understand precisely what role IFNs play in combating disease is of great importance in understanding host responses to

disease. This knowledge would enable more specific therapies to be generated to target particular areas of the IFN pathways important in host defence against disease. Currently, type I IFN is used to treat hepatitis B and C, hairy cell leukemia and chronic myeloid leukemia and more recently IFNβ has been used to treat multiple sclerosis (Ahmed *et al.*, 1999; Zein, 1998). However as the treatment with IFN is dose-limiting due to toxicity, a clearer understanding of how IFN mediates its signals and how these signals are being regulated will be most advantageous in order to make these proteins into more beneficial therapeutic agents.

1.3.1 Viral Infection

Interferons play a major role in host defense against viral infection. Both type I and II IFNs are able to elicit antiviral responses. When a cell is infected, the type I IFNs are one of the first cytokines induced to confer cellular resistance to viral replication. IFN induction occurs firstly due to the direct activation of DRAF which is a DNA binding complex. DRAF is composed of IRF3 and CBP/p300 and induces the production of type I IFNs in order to initiate the transcription of other antiviral genes as previously discussed (Sato *et al.*, 1998; Schafer *et al.*, 1998). IRF3 is expressed constitutively in many cell types and tissues and is activated by dsRNA or viral infection. Activation of IRF3 involves the phosphorylation of the serine and threonine residues which results in translocation to the nucleus, DNA binding and the interaction with p300/CBP, causing an increase in transcriptional activity (Daly & Reich, 1993). As a result, IRF3 plays an integral role in the transcriptional activation of the promoters of the type I IFNs and subsequently the activation of IFNα/β responsive genes either directly or indirectly (Nguyen *et al.*, 1997; Schafer *et al.*, 1998; Yoneyama *et al.*, 1998).

The type I IFNs have been demonstrated to primarily control the initial innate immune response against viral infection, while IFNγ plays a more important role later in the adaptive immune response (Farrar & Schreiber, 1993). However, once IFNs have been induced they exert their actions directly by inducing genes which are able to render individual cells in close proximity to the site of infection

incapable of viral replication, whilst already infected cells are reprogrammed to undergo apoptosis (Tanaka *et al.*, 1998). Indirectly the type I IFNs can induce an antiviral response through the adaptive immune system by activating dendritic and NK cells, which can then function to induce $CD8^+$ T cells along with IFNγ and IL12 production generally later in the course of infection (Nguyen *et al.*, 2000). Therefore the induction of type I IFN serves as a link between innate and adaptive immune responses (Biron *et al.*, 2002; Dondi *et al.*, 2003).

As part of the innate effects of IFNα/β, antiviral genes (ISGs) are transcribed and translated to activate antiviral signaling pathways that block the numerous steps of viral replication. These include the initial entry of the virus into the cell, transcription and translation, maturation of the virus, assembly and virion release (Samuel, 1991; Vilcek *et al.*, 1996). Antiviral ISGs that are known to be commonly induced after viral infection mainly include *2'-5' oligoadenylate synthetase* (*2'-5' OAS*), *dsRNA-dependent protein kinase* (*PKR*) and *Mx* whose proteins functions will be discussed (Samuel, 1991; Vilcek *et al.*, 1996). Therefore it can be assumed that the type I IFNs have an important role in the innate immune response to a viral infection, but also indirectly thorough the induction of other cytokines orchestrates the adaptive immune response.

1.3.2 Interferon Treatments and Therapies

IFNs were discovered over 40 years ago (Issac & Lindemann, 1957), and since then have been shown to be involved in a vast array of molecular mechanisms that maintain homeostasis (Pestka *et al.*, 1987). Therefore it is not surprising that IFNα is one of the most widely used therapeutic cytokines and has been approved to treat more than 14 cancers and viral diseases. Most commonly IFNα2 is used to treat hairy cell leukemia, AIDs related Kaposi's sarcoma (Kirkwood, 2002), chronic myeloid leukemia (Bukowski *et al.*, 2002) and hepatitis C (Perry & Jarvis, 2001). Other α subtypes have been approved to treat other hepatitis subtypes, chronic malignant melanoma, follicullar lymphoma and genital warts. Furthermore, IFNβ is used to treat multiple sclerosis (Strander, 1986; Tamm *et al.*, 1987; Gresser, 1991). However, toxic

effects of IFN give rise to the need for a greater understanding of the IFN signaling system in order to increase the efficacy of treatments.

1.3.2.1 IFN Treatment of Viral Diseases (Hepatitis C)

Chronic *h*epatitis *C v*irus (HCV) has been demonstrated to lead to liver disease, cirrhosis and hepatocellular carcinoma (Fattovich *et al.*, 1997). IFNα induces ISGs which produce biological outcomes that are beneficial to patients suffering from hepatitis C, however only half of the patients treated with IFNα exhibit a sustained viral clearance (McHutchison & Patel, 2002; Tan *et al.*, 2002). During treatment, hours after the initial dose of IFN there is a rapid phase of viral inhibition, thought to result from suppression of viral replication (Neumann *et al.*, 1998). This inhibition of viral replication is believed to enable the immune system to clear the HCV particles from the patient (Neumann *et al.*, 1998). Treatment of patients suffering from hepatitis C will improve as our understanding of the IFN signaling and regulation of signaling within hepatocytes and other target cells increase.

1.3.2.2 Treatment of Autoimmune diseases (MS)

Autoimmunity occurs when there are breaks in signaling cascades resulting in the production of auto-antibodies that attack healthy tissue. *M*ultiple *s*clerosis (MS) is a debilitating condition that can be classified as an inflammatory autoimmune disease. It is characterised by the infiltration of lymphocytes into the cerebral parenchyma and subsequent demyelination of nerves within the central nervous system (Kermode *et al.*, 1990; Kwon & Prineas, 1994).

On a molecular level, MS appears to affect the regulation of IFNβ stimulated genes, causing them to be expressed at low levels. This deceives the immune system into thinking that there is no need for ISG gene induction and thus results in an autoimmune inflammatory disease (Feng *et al.*, 2002). Therefore, this suggests a possible role for IFNβ as a potential therapeutic agent due to its ability to elevate the decreased levels of antiviral proteins (MX and 2'-5' OAS) and IRF to normal levels (Feng *et al.*, 2002).

Currently, IFNβ is used in the treatment of patients that suffer with relapsing forms of MS. IFNβ treatments alter the expression of various genes in order to inhibit autoreactive T cells and MHCII expression which results in antigen presentation within the central nervous system (Lu *et al.*, 1995; Rep *et al.*, 1996). Metalloprotineases are inhibited along with altered expression of cell adhesion molecules (Leppert *et al.*, 1996; Stuve *et al.*, 1996; Calabresi *et al.*, 1997) and increases in immunosuppressive cytokines and inhibition of proinflammatory cytokines result (Noronha *et al.*, 1993; Rudick *et al.*, 1998). However, it has been demonstrated that in MS patients, IFNβ is able to induce high levels of tyrosine phosphorylated STAT1 that results in low levels of SHP1, which terminates STAT signaling. The lack of regulation of the STAT signaling results in elevated levels of IFNγ, Th1 cell activation, generation of auto-antibodies resulting in demyelination of the central nervous system (David *et al.*, 1995; Massa *et al.*, 2000; Zang *et al.*, 2000).

1.3.2.3 Treatment of Cancers (Hairy Cell Leukemia)

IFNα has been approved for use in the treatment of some hematological cancers such as hairy cell leukemia, chronic myeloid leukemia and some B and T cell lymphomas (Vedantham *et al.*, 1992). Hairy cell leukemia is a chronic lymphoproliferative disorder of B cells. Normal B cell growth and differentiation have defined pathways that malignancies can form in at any stage producing a pre-plasmatic B cell expressing CD25 which is known as a hairy cell, hence hairy cell leukemia (Gutterman, 1994). IFNα has been used to treat this cancer with success as the decreased levels of peripheral blood cells and platelets increase (Quesada *et al.*, 1984), with the immune status improving with hairy cell numbers decreasing (Lepe-Zuniga *et al.*, 1987).

More recent studies have demonstrated that there are no IFNα transcripts in PBMC of HCL untreated patients due to a reduced capacity for IFN production in B and T cells (Shehata *et al.*, 2000). With treatment of recombinant IFNα, transcript levels increase as does the production of endogenous IFNα as the recombinant primes for endogenous IFNα production (Shehata *et al.*, 2000).

Exposure of hairy cells to IFNα was demonstrated to increase TNFα secretion resulting in apoptosis. Using antibodies to neutralize TNFRI and TNFRII, the IFNα induced apoptosis was removed, demonstrating than in the presence of IFNα, TNFα induces apoptotic pathways to decrease the hairy cell numbers (Baker *et al.*, 2002). Most importantly with the treatment of IFNα for HCL, low doses of IFNα are effective, therefore making it a beneficial therapeutic agent.

1.4 CYTOKINE RECEPTORS

Given the importance of disease pathogenesis and treatment, the mechanisms of activation of IFN signaling through their receptor are important. Cytokine transmembrane receptors have been characterised into two groups, class I and class II, based on the general structural characteristics of their extracellular domains (Bazan, 1990; Heldin, 1995; Kishimoto *et al.*, 1994; Thoreau *et al.*, 1991). These receptors are able to function in combination with cytoplasmic signaling molecules/pathways to transduce signals that activate the transcription and translation of specific proteins hence producing biological outcomes (Moore *et al.*, 1993).

The majority of cytokine receptors are class I receptors, which drive signaling through pathways such as the JAK/STAT pathway. The class I receptors possess four conserved cysteine residues and a WSxWS motif and are predominantly receptors for haematopoietic cytokines such as IL2, IL3, IL4, IL6, IL7, G-CSF, GM-CSF and erythropoietin (Fiorentino *et al.*, 1991; Hsu *et al.*, 1992; Bogdan *et al.*, 1991). The class II receptors form a smaller group which comprise the type I and II IFN receptors, IL10 receptor (Ho *et al.*, 1993), tissue factor (TF) receptor, IL10R2 (CRF2-4), IL22R1 (CRF2-9), IL22BP (CRF2-10), IL20R1 (CRF2-8) and IL20R2 (CRF2-11) (Kotenko, 2002). These receptors also possess four conserved cysteine residues that have a different alignment to the class I receptors (Bazan, 1990; Thoreau *et al.*, 1991). Another distinguishing factor is the lack of the conserved WSxWS motif of the class I cytokine receptors (Bazan, 1990). The class II receptors lack intrinsic kinase activity, therefore are prime candidates for JAK association. Indeed, the

majority of signal transduction elicited by these receptors is by activation of the JAK/STAT pathway (Kotenko & Pestka, 2000).

Each of the individual class II cytokine receptor subunits are comprised of two tandem fibronectin type III domains (D200), with the exception of IFNAR1, which contains four. Each of the receptor complexes are thought to be made up of a primary ligand binding chain and an accessory chain both required for optimal signaling to occur (Kotenko & Pestka, 2000).

It is important to note that along with transmembrane receptors that have been referred to, there are also soluble receptors (e.g.: IFNAR2a and soluble IL6). These soluble receptors have the capability to act as both inhibitors and activators making them important players in both negative and in some cases positive regulation (Hardy *et al.*, 2001).

1.4.1 Type I Interferon Receptors

All type I IFNs bind to the *i*nterferon alpha *r*eceptor designated as IFNAR. The *Ifnar1* and *Ifnar2* genes are located on human chromosome 21 and murine chromosome 16 (Novick *et al.*, 1994; Lutfalla *et al.*, 1995; Kotenko & Pestka, 2000). This receptor is comprised of two chains IFNAR1 and IFNAR2, and is present at low numbers on the cell surface of all cell types (Novick *et al.*, 1994; Uzé *et al.*, 1990). Both of the subunits IFNAR1 and IFNAR2 lack intrinsic kinase activity and are preassociated with JAK family members, IFNAR1 with TYK2 and IFNAR2 with JAK1 in the absence of ligand binding (Stark & Kerr, 1992; Richter *et al.*, 1998; Kotenko *et al.*, 1999).

The huIFNAR1 chain was cloned and identified by transfecting murine cells with human DNA and selecting for species specific human type I IFN responsive cells (Uzé *et al.*, 1990). Both the human and murine IFNAR1 component is a 110 kDa glycoprotein, and is present in only one isoform (Lutfalla *et al.*, 1992; Hwang *et al.*, 1995). It has distinct features in the extracellular domain which make it different from the rest of the class II cytokine receptor family. The D200 domain that is comprised of two fibronectin type III domains is repeated in the

extracellular portion of the IFNAR1 receptor chain (Figure 1.3). There is conflicting data on the role that the IFNAR1 subunit is believed to play. Some data suggests that it is the accessory subunit which regulates the recognition of the different type I IFNs by the IFNAR complex rather than playing a primary part in ligand binding, whereas other data implies that IFNAR1 does play a role in binding (Cleary *et al.*, 1994; Cook *et al.*, 1996; Cutrone *et al.*, 1997). However, these discrepancies are most likely due to cell specificity (Hwang *et al.*, 1996).

The IFNAR2 chain exists in 3 isoforms in humans (Novick *et al.*, 1994; Lutfalla *et al.*, 1995) and only 2 in mice (Kim *et al.*, 1997; Owczarek *et al.*, 1997). These multiple isoforms have been shown to result from alternate splicing of the respective *Ifnar2* genes. This splicing event results in one of the following being translated: a full length transmembrane form (IFNAR2c), a truncated transmembrane form (IFNAR2b) which appears only in the human, or a soluble form (IFNAR2a) (Figure 1.3) (Lutfalla *et al.*, 1995; Novick *et al.*, 1994; Kim *et al.*, 1997; Owczarek *et al.*, 1997). Each of these splice variants have identical extracellular domains and are able to bind type I IFN (Mogensen *et al.*, 1999; Prejean & Colamonici, 2000). Experiments utilising human U5A cells, null for IFNAR2 and insensitive to type I IFNs demonstrated that only through the re-expression of IFNAR2c and not IFNAR2b, that IFNAR2 was required for signaling through a functional type I IFN receptor (Lutfalla *et al.*, 1995). More specifically, within the intracellular domain of IFNAR2c there are two proximal tyrosines which are also present on IFNAR1 which are required for efficient type I IFN signaling to occur (Nadeau *et al.*, 1999; Kotenko *et al.*, 1999).

Mice with a null mutation in the *Ifnar1* (Müller *et al.*, 1994; Hwang *et al.*, 1995) and *Ifnar2* (Hertzog, unpublished) genes have been generated. The generation of these mouse models has proven to be useful tools in defining type I IFN signaling. Cells from mice in which the *Ifnar1* gene has been disrupted are able to bind IFN, indicating that IFNAR2 has the ability to bind IFN to some extent independently of IFNAR1, however less than in the wild-type controls, implying that IFNAR1 contributed to ligand binding (Owczarek *et al.*, 1997). In the absence of IFNAR1, the IFNAR2 receptor in some cells appear to be unable to

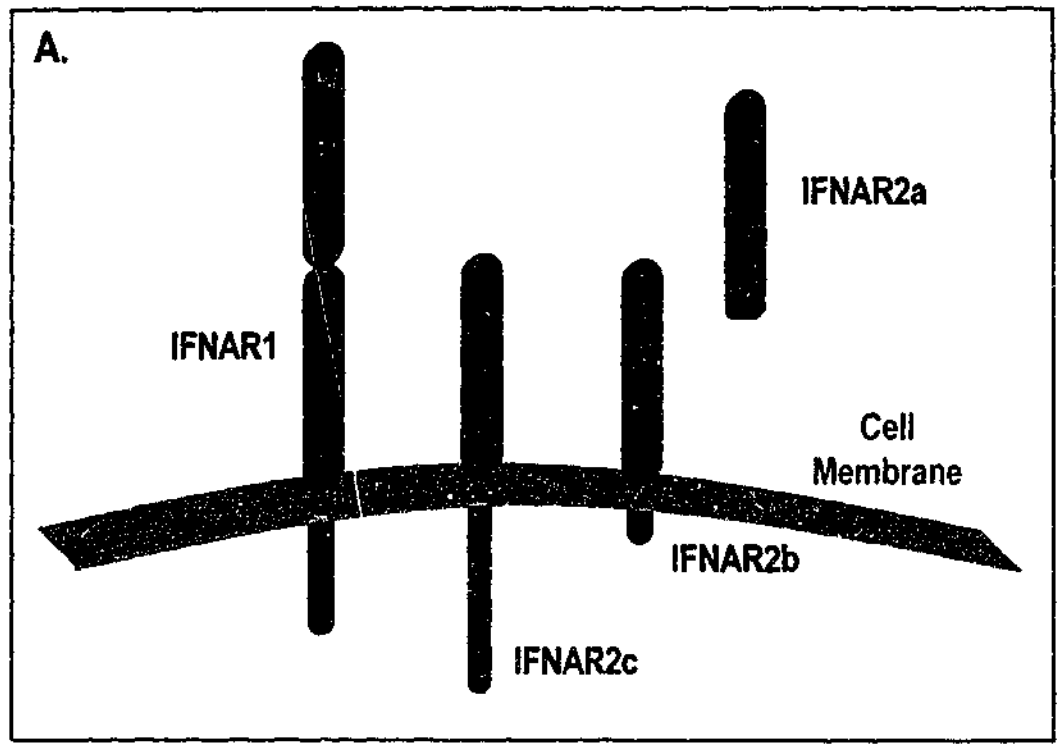


Figure 1.3: Type I IFN Receptor Subunits.

A. The human type I IFN receptor subunits IFNAR1, IFNAR2c, truncated IFNAR2b and the soluble IFNAR2a. Cysteine residues are shown as horizontal lines in the extracellular domains.

B. The murine type I IFN receptor subunits IFNAR1, IFNAR2c and the soluble IFNAR2a. Cysteine residues are shown as horizontal lines in the extracellular domains.

effectively transduce signals (Hwang *et al.*, 1995; Müller *et al.*, 1993; Owczarek *et al.*, 1997). The generation of the *Ifnar1*$^{-/-}$ has highlighted the importance of this receptor subunit in antiviral responses and haematopoiesis (Hwang *et al.*, 1995; Müller *et al.*, 1994). Extensive analyses of the haematopoietic lineages of each of the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice demonstrate that both of the receptor knockout models reproducibly present with slightly different phenotypes. The *Ifnar1*$^{-/-}$ mice are ill equipped to generate either antiviral or antiproliferative responses to exogenous IFNα/β (Hwang *et al.*, 1995). Cells of the myeloid lineage proved to be abnormal in number while all other parameters of haematopoiesis were comparable to wild-type mice. Interestingly the *Ifnar2*$^{-/-}$ mice also fail to respond to viral infection making them highly susceptible to infection, albeit not to the same extent as the *Ifnar1*$^{-/-}$, while the abnormalities found in these mice were specific to T lymphocytes and the thymus. The *Ifnar2*$^{-/-}$ mice have an increased thymus size with an increase in the number of CD8^{+} cells resulting from a malfunction in the apoptotic signaling of these cells within the thymus (Hertzog, unpublished). Similar to the *Ifnar1*$^{-/-}$ mice, all other parameters of haematopoiesis were normal in the *Ifnar2*$^{-/-}$ mice.

It has become apparent from these studies that there may be distinct roles for the two chains of the type I IFN receptor complex in signal transduction. Mice which have a disrupted *Ifnar1* gene have defects in cells of the myeloid lineage (Hwang *et al.*, 1995), whilst *Ifnar2*$^{-/-}$ mice have defects in cells in the lymphoid lineage (Hertzog *et al.*, unpublished). This suggests that in organs or tissues that myeloid cells populate, the IFNAR1 chain of the receptor plays a particularly important role, and thus the absence of this chain results in defective cells of this lineage. Likewise in organs or tissues which lymphoid cells populate, the IFNAR2 chain may have an important regulatory role which is lost in the absence of a functional IFNAR2 chain. Importantly, a double knockout of the two chains of the receptor is fetal lethal (Hertzog *et al.*, unpublished) suggesting that neither of the two single knockout models result in the total loss of IFNAR activity. Again this implies that a residual degree of signaling remains in the two knockouts, probably utilising different signal transduction cascades.

1.4.2 Type II Interferon Receptors

The type II IFN receptor is a multicomponent structure designated as IFNGR, composed of IFNGR1 and IFNGR2 (Figure 1.4). The *Ifngr1* gene is located on human chromosome 6 (Rashidbaigi *et al.*, 1986) and on mouse chromosome 10 (Mariano *et al.*, 1987; Kozak *et al.*, 1990), while the *Ifngr2* gene is situated on human chromosome 21 (Jung *et al.*, 1987) and on mouse chromosome 16 (Hibino *et al.*, 1991). These subunits, expressed ubiquitously on different cell types, act as dimers. Two IFNGR1 chains are involved in binding the ligand dimer while the two IFNGR2 chains are recruited as accessory chains, the presence of which are necessary for signaling to occur (Jung *et al.*, 1987; Aguet *et al.*, 1988; Valente *et al.*, 1992; Kotenko *et al.*, 1995).

Both IFNGR1 and 2 lack intrinsic kinase motifs, as do all class II cytokine receptors and, like the IFNAR complex, are associated with JAKs. JAK1 is associated with IFNGR1 prior to ligand binding (Igarashi *et al.*, 1994) while JAK2 is associated with the intracelluar domain of IFNGR2 directly in the absence of IFNγ (Kontenko *et al.*, 1995). IFNγ induction activates both of the preassociated JAKs. Studies utilising *Jak1* null cells (U4A), have implied that some IFNγ responses are induced, whereas *Jak2* negative cells (γ2A) fail to respond to IFNγ (Briscoe *et al.*, 1996). These results imply that JAK1 plays a more structural role, with JAK2 playing a catalytic role (Kontenko *et al.*, 1995). These findings are further supported by studies involving *Ifngr1* and *Ifngr2* knockout mice. Mice null for *Ifngr2* are unable to respond to IFNγ and hence are deficient in IFNγ mediated immune responses (Lu *et al.*, 1998). JAK2 is unable to be activated in the IFNGR complex as IFNGR2 is no longer present to recruit JAK2 to the receptor unit. Mice lacking *Ifngr1* are susceptible to viral infection and are unable to respond to IFNγ. Therefore the presence of both JAK1 and JAK2 is required for the assembly of the IFNGR in order to respond to IFNγ.

The IFNGR1 subunits are believed to form an inactive IFNγ receptor, which requires the recruitment of IFNGR2 subunits that with their associated JAK2 proteins initiate signal transduction (Kotenko *et al.*, 1995, 1996). Within the

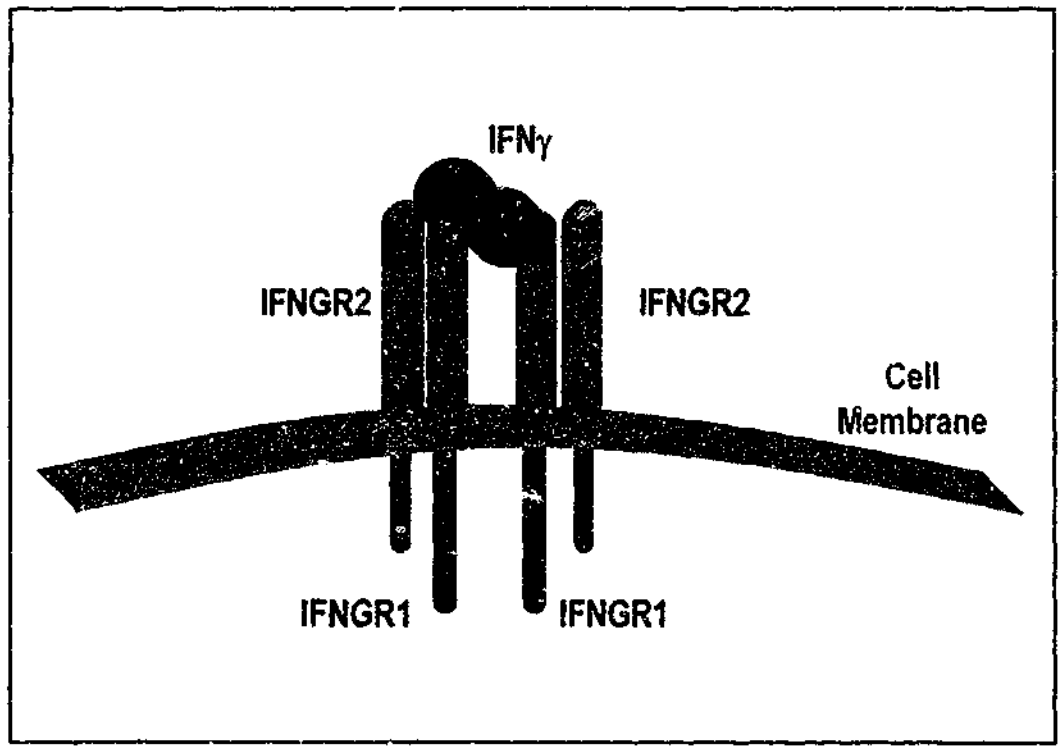


Figure 1.4: Type II IFN Receptor.

The type II IFN, IFNγ binds as a homodimer to a separate receptor complex comprising of two chains of IFNGR1 and two chains of IFNGR2. The receptors do not associate with each other until ligand binding.

intracellular domain of the IFNGR1 there are two important domains which have been elucidated as being important for signal transduction to occur (Cook *et al.*, 1992; Farrar *et al.*, 1992). Farrar *et al.* (1991) demonstrated that the domain proximal to the transmembrane domain is necessary for internalisation of the receptor-ligand as well as for biological responses. This second domain near the carboxyl terminus becomes the STAT1 docking site following receptor ligand interaction and subsequent activation (Greenlund *et al.*, 1994). Thus, in a similar manner to the IFNAR system the IFNGR pathway requires the presence of multiple components in order to initiate activation of the downstream effects.

1.4.3 Soluble Receptors

There are many transmembrane receptors that have associated soluble receptors. One such example of this is the IFNAR-associated soluble type I IFN receptor, IFNAR2a. Soluble receptors are generally formed as a result of alternative splicing of the transmembrane receptor gene or proteolytic cleavage of a cell surface receptor (Rose-John & Heinrich, 1994). Soluble receptors are able to function as either agonists or antagonists depending on the biological situation (Kim & Maniatis, 1996; Hardy *et al.*, 2001).

The soluble type I IFN receptor is generated as a splice variant from the *IFNAR2/Ifnar2* gene. Studies using the *Ifnar2* knockout mouse cells have highlighted that in the absence of the IFNAR2c chain, exogenous soluble IFNAR2a receptor chain can form a complex with IFNα or IFNβ and IFNAR1 to generate an antiproliferative response in primary thymocytes (Hardy *et al.*, 2001). In contrast, when both IFNAR1 and IFNAR2 are present such as in murine L929 cells, primary thymocytes and murine embryo fibroblasts, recombinant IFNAR2a can inhibit type I IFN signaling (Hardy *et al.*, 2001). Thus IFNAR2a has been demonstrated in an artificial system to act in two opposing ways, firstly as an IFN signaling receptor and secondly as an inhibitor of IFN signaling. This diversity in function exhibited by soluble IFNAR2 could be explained by the different abundances of the soluble versus transmembrane isoforms of IFNAR2 in different cells and tissues.

1.5 SIGNAL TRANSDUCTION COMPONENTS

Signal transduction is the process initiated by ligand binding to activate proteins to transfer messages from outside the cell through the cytoplasm to the nucleus. This usually results in either an increase or a decrease in gene expression and subsequent protein synthesis.

1.5.1 Janus Kinases (JAKs).

The *Janus kinase* (JAK) family of cytoplasmic tyrosine kinases are well-characterised core components of IFN signaling from cell surface receptors, which lack intrinsic kinase activity. In the mammalian system, there are four JAKs, including JAK1, JAK2, JAK3 and TYK2 that preferentially preassociate with cytokine receptors. When ligands bind their cognate receptors, each of the JAKs have been demonstrated to initiate signaling cascades (Darnell *et al.*, 1994; Ihle, 1995; Yeh & Pellegrini, 1999).

Initially the JAKs were discovered as novel protein tyrosine kinases that associated with cytokine receptors (Ihle *et al.*, 1995; Pellegrini *et al.*, 1997). More specifically, TYK2 was isolated using a low stringency hybridisation screen of a T cell cDNA library with *c-fms* catalytic domain (Krolewski *et al.*, 1990). In contrast JAK1, JAK2 and JAK3 were cloned utilising a PCR strategy using primers corresponding to conserved motifs within the catalytic domain of *Tyk2* (Wilks *et al.*, 1991; Harpur *et al.*, 1992; Rane & Reddy, 1994). The common roles that the JAKs play in the IFN signaling pathways have since been elucidated by the generation of mutagenised cell lines that were resistant to IFNs effects (Velazquez *et al.*, 1992; Feng *et al.*, 1997; Zhou *et al.*, 1997; Darnell *et al.*, 1994; Darnell, 1997). Once activated by ligand binding and subsequent trans-phosphorylation, the JAKs have three functions, firstly, they phosphorylate their associated receptors on tyrosine residues (Colamonici *et al.*, 1994). The receptor phosphorylation forms a STAT docking site enabling the recruitment of specific latent STAT proteins to the activated receptor complex. The second function of JAK is the subsequent phosphorylation of tyrosine residue on the STAT protein (Darnell *et al.*, 1994; Stahl *et al.*, 1995).

Thirdly, the JAKs are able to assist in the formation/stability of the receptor complex in the membrane, generating the highest affinity for its respective ligand, as TYK2 does for IFNAR1 (Velasquez *et al.*, 1995). While most of the literature discusses the JAKs in terms of phophorylating STAT proteins, they also associate/activate GRB2, SHP2, VAV and STAM resulting in the activation of multiple pathways (Yin *et al.*, 1997; Chauhan *et al.*, 1995; Matsuguchi *et al.*, 1995; Takeshita *et al.*, 1997). The JAKs have also been shown to bind to SOCS family members once activated, acting as an adapter between receptor subunits and SOCS proteins which, target proteins for proteosomal degradation (Yasukawa *et al.*, 1999; Nicholson *et al.*, 1999).

JAK proteins range in size from 110 kDa to 140 kDa, possess a C-terminal protein kinase domain (JH1), an adjacent kinase or kinase-related domain (JH2) plus a further five domains extending towards the N-terminus (JH3-7) (Harpur *et al.*, 1992) (Figure 1.5). The JH1 domain, which is the tyrosine kinase domain, has been reported to require the pseudodomain JH2, in order to elicit its catalytic activity (Yeh *et al.*, 2000; Velazquez *et al.*, 1995). The remaining five domains JH3-7 have been implicated in receptor association. The chromosomal locations of each of the JAKs have been identified in humans as chromosomes 1 for *JAK1*, 9 for *JAK2* and 19 for *JAK3* and *TYK2* (Krowlewski *et al.*, 1990; Pritchard *et al.*, 1992; Riedy *et al.*, 1996). In mice, *Jak1*, *Jak2*, *Jak3* and *Tyk2* localize to chromosome 4, 19, 8 and 9 respectively (Gough *et al.*, 1995; Kono *et al.*, 1996; Mouse Genome Informatics Scientific Curators, 2002).

JAK1, JAK2 and TYK2 are expressed ubiquitously and bind to numerous cytokine receptor subunits, whereas JAK3 appears to be restricted to cells of haematopoietic origin (Ihle, 1995). The functional importance of the JAK family of kinases in mediating cytokine signaling was elucidated through a series of experiments utilising mutant cell lines lacking each of the individual *Jaks*. The disruption of each of the *Jaks* to generate mouse knockout models further elucidated a specificity of JAK1, 2 and TYK2 but not JAK3 to induce cellular responses to IFNα/β and IFNγ (Velazquez *et al.*, 1992; Müller *et al.*, 1993; Watling *et al.*, 1993).

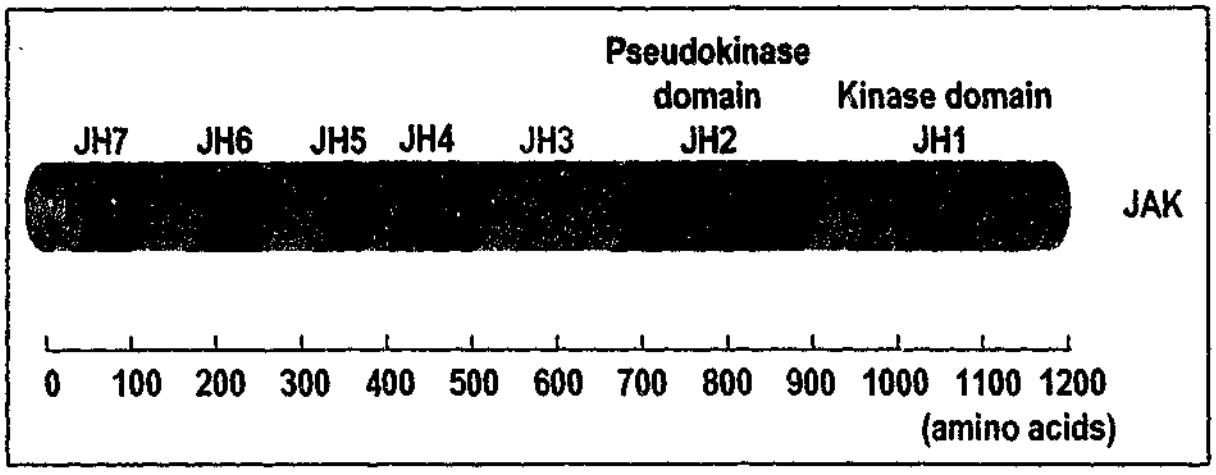


Figure 1.5: JAK Protein Structure.

The JAK proteins share 7 regions of homology and have been denoted as JAK homology domains (JH) 1-7. JH1 is the kinase domain at the C-terminal end, JH2 is a pseudokinase domain while the remaining JH3-7 have been implicated in receptor binding.

Each of the JAKs require the presence of one other functional JAK in order to initiate signaling cascades. It is the intermolecular tyrosine phosphorylation of the JH1 domain of these JAKs that initiate signaling rather than intramolecular phosphorylation (Darnell, 1997). It was demonstrated using *Jak1* mutant cells (U4A) that cytokines which utilise class II receptors, the γ common chain of the IL12 receptor and the gp130 subunit were unable to signal (Rodig *et al.*, 1998). In particular for IFNs, it was shown that JAK1 was required for signaling to occur in response to both IFNα/β and IFNγ as no IFN (both type I and II) signaling was observed by means of ISG induction in *Jak1* mutant cell lines (Kotenko & Pestka, 2000; Müller *et al.*, 1993). However, by reconstituting *Jak1* into the U4A cell line, normal signaling by all mentioned cytokine receptors was restored. The generation of the *Jak1*$^{-/-}$ mouse substantiated the *in vitro* data in that these mice were unresponsive to cytokines utilising all class II cytokine receptors (including both type I and II IFN), the γ common chain receptor and gp130 receptor (Rodig *et al.*, 1998). The lack of signaling in response to such a large number of cytokines contributed to these mice exhibiting perinatal lethality resulting from both defective neural function and lymphoid development (Rodig *et al.*, 1998). It is clear particularly from data derived from *Jak1* deficient systems that JAK1 plays a central role in many signaling pathways.

The *Jak2* mutant cell line (γ1A) failed to respond to IFNγ (Neubauer *et al.*, 1998; Parganas *et al.*, 1998) however responded normally to IFNα/β (Watling *et al.*, 1993). This implied that JAK2 was not involved in type I IFN signaling but was necessary for IFNγ signaling. However, experiments where functional *Jak2* was re-expressed in γ1A cells, IFNγ signaling was restored (Watling *et al.*, 1993). The generation of the *Jak2*$^{-/-}$ mouse again supported *in vitro* data, in that fibroblasts derived from these embryos fail to respond to IFNγ, but do respond to IFNα/β. Phenotypically the *Jak2*$^{-/-}$ mice suffer embryonic lethality resulting from the absence of erythropoiesis and hence exhibit anaemia due to the failure of erythropoietin signaling (Neubauer *et al.*, 1998; Parganas *et al.*, 1998; Aringer *et al.*, 1999). This data, along with the *Jak1* mutant studies indicated that although sequence/structural similarity exists between these proteins, there is little functional redundancy within this family, at least within the IFN system.

The third kinase that associates with the IFN receptors is TYK2. More specifically it associates with IFNAR1 (Uze *et al.*, 1990; Yan *et al.*, 1996b) and positively influences ligand binding to the receptor complex. Utilising *Tyk2* deficient cells (11,1), it was shown that the amino terminal region and the kinase-like domain of TYK2 were required for high affinity binding of type I IFNs as a reduced uptake of labeled IFN was exhibited. When these cells were reconstituted with active TYK2, binding of IFNα to the receptor returned to normal levels (Pellegrini *et al.*, 1989; Velazquez *et al.*, 1995). TYK2 deficient cell lines were still able to respond to type I IFNs, albeit suboptimally (Karaghiosoff *et al.*, 2000; Shimoda *et al.*, 2000). In human cells, residual responses to IFNβ were detected, however there was no response to IFNα (Velazquez *et al.*, 1995). However, TYK2 does appear to be required for structural integrity of the IFNAR signaling complex as demonstrated when re-expression of inactive TYK2 in 11,1 cells restored a higher level of signaling in response to IFNα/β compared to the removal of TYK2 (Gauzzi *et al.*, 1996). The *Tyk2*$^{-/-}$ mice display no overt developmental abnormalities, they are unable to respond to small amounts of IFNα/β but can respond to high concentrations of IFNα/β and have a defect in IL12 induced T cell function (Shimoda *et al.*, 2000). Recent studies have demonstrated that *Tyk2*$^{-/-}$ mice are resistant to septic effects of LPS treatment, making *Tyk2* a component of the LPS signaling pathways (Karaghiosoff *et al.*, 2003). The fact that there is residual signaling occurring in response to type I IFNs in *Tyk2* deficient cells suggests that unlike the JAK proteins there is some TYK2 functional redundancy, as IFN signaling is not dependent on fully functional TYK2 (Shimoda *et al.*, 2000).

JAK3 differs from the other three tyrosine kinases and has little effect on IFN signaling. Rather JAK3 specifically interacts with the γ common chain in response to IL2, IL4, IL7, IL9 and IL15 signaling. Cells deficient for *Jak3* are therefore deficient in γ common chain signaling, especially in response to IL2 (Oakes *et al.*, 1996). Therefore it is not surprising that JAK3 plays an essential role in γ common chain dependant lymphoid development (Di Santo *et al.*, 1995; Cao *et al.*, 1995; Thomis *et al.*, 1995; Nosaka *et al.*, 1995; Park *et al.*, 1995). *Jak3*$^{-/-}$ mice suffer from severe immunodeficiency due to a marked reduction in

the number of T and B cells (Nosaka *et al.*, 1995; Park *et al.*, 1995; Thomis *et al.*, 1995) and exhibit a phenotype similar to human severe combined immunodeficiency (SCID) resulting from *JAK3* mutations (Macchi *et al.*, 1995; Russell *et al.*, 1995). These immunodeficiencies result from the impaired signaling of the numerous interleukins heavily involved in lymphoid development (Russell *et al.*, 1995). Unlike JAK1, JAK2 and TYK2, JAK3 appears to have a role in the pathways initiated from the γ common chain, with the *Jak3*$^{-/-}$ mice exhibiting little effect on the IFN system.

Therefore it could be concluded that both JAK1 and JAK2 are required for IFNγ signaling pathways, while for functional IFNα/β signaling to occur, JAK1 and TYK2 are required both for signaling and optimal receptor stability in the membrane (Shuai *et al.*, 1993).

1.5.2 Signal Transducers and Activators of Transcription (STATs)

The STAT proteins, which form an integral part of the majority of cytokine signaling, were first discovered through investigations into IFN signaling, more specifically by an investigation of transcription factors which bound to promoter regions of *I*FN *s*timulated *g*enes (ISGs) (Darnell *et al.*, 1994). Subsequently, seven structurally and functionally related STATs have been identified, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. All STATs are expressed ubiquitously, with the exception of STAT4, which is expressed only in the brain, heart, spleen, blood cells and testis (Fu *et al.*, 1992; Zhong *et al.*, 1994; Akira *et al.*, 1994; Yamamoto *et al.*, 1994; Wakao *et al.*, 1994; Mui *et al.*, 1995; Azam *et al.*, 1995; Lin *et al.*, 1996; Hou *et al.*, 1994). Generally, STATs are involved in signaling from the cell membrane receptors following recruitment to activated receptor complexes to the nucleus. Once in the nucleus, they have been demonstrated to mediate transcriptional regulation in response to numerous growth factor and cytokine families, one of which is the IFN family (Darnell *et al.*, 1994; Darnell, 1997).

Genetic mapping of the STAT gene family has demonstrated that in both human and mouse, the *STAT/Stat* genes localise to three separate clusters on different chromosomes. Both *STAT1/Stat1* and *STAT4/Stat4* cluster together on human chromosome 2 and mouse chromosome 1. *STAT2/Stat2* and *STAT6/Stat6* make up the second group localised to human chromosome 12 and mouse chromosome 10, with the remaining *STAT3/Stat3*, *STAT5a/Stat5a* and *STAT5b/Stat5b* constituting the final group localised to human chromosome 17 and mouse chromosome 11 (Copeland *et al.*, 1995; Lin *et al.*, 1995). The similarities in the clustering and structure of both human and mouse *STAT/Stat* genes, suggest that each of the STATs have most likely evolved as a result of a number of tandem duplications during the evolution of multicellular organisms (Hou *et al.*, 1996; Yan *et al.*, 1996b).

The STAT proteins range in size from 750 to 850 amino acids and share a similar structure of 6 domains. The domains include the amino terminal domain (NH2) which has been implicated in interactions with receptor domains and other transcription factors such as CBP/p300 and members of the PIAS family (Horvath, 2000; Leung *et al.*, 1996; Murphy *et al.*, 2000; Shuai, 2000). The coiled-coiled domain (CCD) forms an interactive surface to which other helical proteins such as IRF9 are able to bind (Horvath *et al.*, 1996). The *DNA binding domain* (DBD) makes up the region that recognises DNA sequences within promoter regions of STAT induced genes. STAT1 dimers can interact with gamma activated sequence (GAS) elements in both IFNγ and IFNα/β signaling. STAT2 is the only STAT that does not interact with DNA (Qureshi *et al.*, 1995). The linker domain connects the DBD to the SH2 domain and has been implicated in transcriptional regulation through interactions with the SH2 domain, which is a docking site for the transcription factors that the STATs interact with (Chen *et al.*, 1998; Yang *et al.*, 1996). The SH2 domain contains the tyrosine activation motif that is integral in signaling. The SH2 domain plays an important role in 3 events involved in STAT signal transduction, these include recruitment to the receptor chain, interaction with the activated JAKs and the subsequent hetero- or homodimerisation upon phosphorylation (Barahmand-Pour *et al.*, 1998; Gupta *et al.*, 1996; Shuai *et al.*, 1994). The final domain is the transcriptional activation domain (TAD) located at the carboxyl

terminus. This domain is poorly conserved between the STAT proteins and thus gives each of the STATs some specificity (Figure 1.6). Each of the STATs harbour conserved domains as discussed which suggests that they each carry out similar functions albeit within different signaling pathways activated by numerous stimuli.

Latent STAT proteins are localised to the cytoplasm of cells until they are activated through receptor complex-mediated tyrosine phosphorylation. The STATs are predominantly involved in pathways that utilise receptor subunits lacking in intrinsic kinase activity, and hence employ the use of JAKs. However, STATs have been shown to be activated by receptor tyrosine kinases EGF-R and CSF1-R (Leonard & O'Shea, 1998; Schindler & Strehlow, 2000) as well as non-receptor kinases such as src and abl (Bromberg & Darnell, 2000).

Members of the STAT family have six essential roles, (1) they bind via their SH2 domains to tyrosine residues on the activated receptor complex that have been phosphorylated by receptor associated JAKs. (2) Each STAT protein possesses a conserved tyrosine residue that sits approximately 700 residues away from the N-terminus, and acts as the substrate for JAK phosphorylation (Horvath & Darnell, 1997). In the case of IFNα/β signaling, it is thought that STAT2 binds to the receptor subunit then, once phosphorylated, forms a docking site for STAT1 (Li *et al.*, 1997). (3) Once bound the STATs themselves become phosphorylated on tyrosine residues (Y701 STAT 1, Y688 STAT 2) and in some instances STAT1 is also serine phosphorylated on residue S727 (Wen *et al.*, 1995; Kovarik *et al.*, 1998; Goh *et al.*, 1999; Uddin *et al.*, 2000a). (4) Dimerisation is the next step that occurs between the SH2 domain of one STAT protein and the phosphorylated tyrosine of another. (5) Bivalent interactions stabilise these structures and thus give rise to the phosphorylated STATs forming hetero- and homodimers rather than staying bound to the receptors via the SH2 domain (Leonard & O'Shea, 1998). Following dimerisation, (6) the newly formed transcription factor is able to translocate to the nucleus where they are able to bind DNA resulting in the modulation of gene expression.

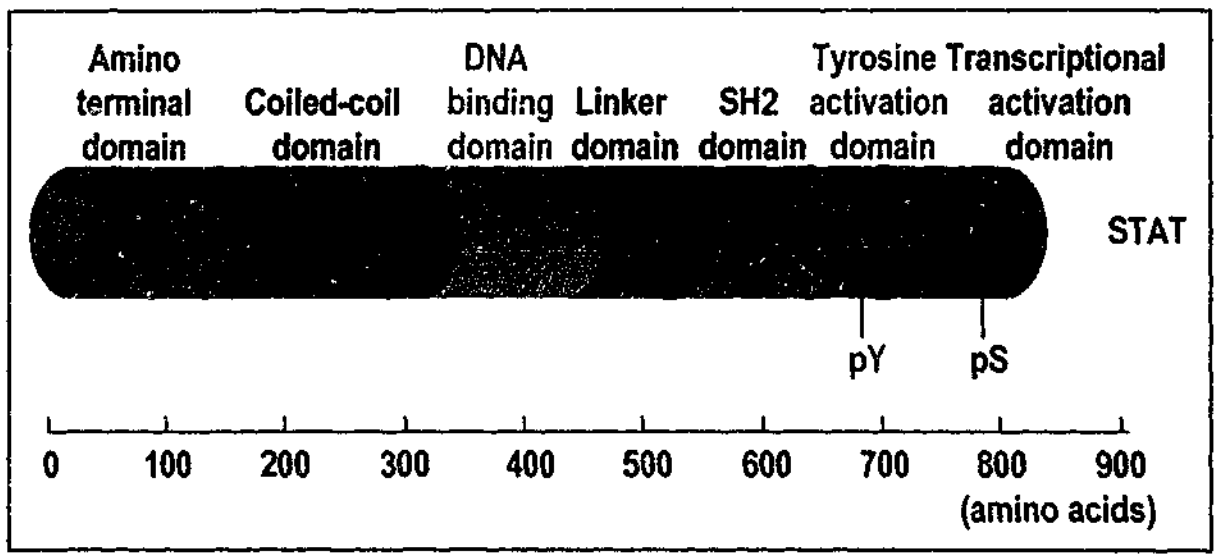


Figure 1.6: STAT Protein Structure.

The STAT proteins share 6 regions of homology with the amino terminal region, coiled-coil, DNA binding, linker, SH2, tyrosine activation and a transcriptional activation domain at the C-terminal end.

The functions of the STAT proteins have been examined with the use of both mutant cell lines and knockout models. Studies using mutant *STAT1* cells (U3A) (McKendry *et al.*, 1991) have demonstrated that STAT1 is required for both type I and II IFN signaling, evidence which was supported by data resulting from the generation of the *Stat1*$^{-/-}$ mouse (Durbin *et al.*, 1996). Numerous ligands activate STAT1 *in vitro* however, the *Stat1*$^{-/-}$ mouse demonstrated a lack of IFN signaling, in particular immune responses to viruses and bacteria (Durbin *et al.*, 1996; Meraz *et al.*, 1996). These mice are however able to respond normally to several other cytokines and growth factors which activate STAT1 *in vitro* (Larner *et al.*, 1993; Fu & Zhang, 1993; Finbloom & Winestock, 1995; Wen *et al.*, 1995) indicating that *in vivo* the lack of STAT1 is necessary for IFN signaling but not for other signaling pathways which are activated *in vitro* (Meraz *et al.*, 1996).

STAT2 is a unique family member in that there is not a high degree of homology between the human and mouse forms, with the mouse form being larger than the human (Park *et al.*, 1999). However, the signaling of STAT2 remains similar in both species (Park *et al.*, 1999). Mutant cell lines lacking functional STAT2 (U6A) were used to discover that STAT2 is required for ISGF3 formation in response to IFNα/β and therefore defects were observed in induction of ISGF3 target genes. Even though STAT2 is not directly involved in the activation of GAS driven genes, there was a lack of STAT1 homodimer formation in response to IFNα/β in the *Stat2* deficient cells (Leung *et al.*, 1995). These findings indicated that STAT2 plays a structural role in serving as a docking site for STAT1 activation by type I IFNs (Leung *et al.*, 1995; Farrar *et al.*, 2000). The phenotype exhibited by *Stat2*$^{-/-}$ mice involves unresponsiveness to type I IFN, a high susceptibility to viral infection, and defects in macrophages and T cell responses. This evidence supported a role for IFNs in innate and acquired immune responses. Type I IFNs were unable to induce ISGF3 driven or GAS driven genes in *Stat2*$^{-/-}$ mice (Park *et al.*, 2000). However, STAT1 homodimerisation in response to IFNγ was not affected, implying STAT2 is not involved in IFNγ signaling (Park *et al.*, 2000), but is required for type I IFN induced ISGF3 signaling along with STAT1 homodimer signaling as STAT2

appears to act as a docking site for STAT1 phosphorylation (Leung *et al.*, 1995).

The remaining 5 STATs are predominantly involved in signaling in response to cytokines other than IFN. Each of these STATs have been knocked out and the null mice are viable, with the exception of the *Stat3*$^{-/-}$ which is embryonic lethal. However, studies have demonstrated that STAT1:STAT3 heterodimers can form in response to IFNα (Owczarek *et al.*, 1997). Although the null mice are viable, they do exhibit phenotypic alterations specific to each STAT. *Stat4*$^{-/-}$ mice lack responsiveness to IL12 causing them to be unable to influence Th1 cellular responses (Kaplan *et al.*, 1996; Thierfelder *et al.*, 1996). *Stat5a*$^{-/-}$ mice display abnormal adult mammary gland development and lactogenesis due to deficient prolactin signaling (Liu *et al.*, 1997). *Stat5b*$^{-/-}$ mice suffer a decreased responsiveness to growth hormone (Udy *et al.*, 1997). Finally, *Stat6*$^{-/-}$ mice display a complete loss of IL4 induced Th2 responses (Takeda *et al.*, 1996; Shimoda *et al.*, 1996). The use of knockout models has highlighted the importance of the STAT proteins and defined the specificity in some of their actions.

1.5.3 Interferon Regulatory Factors (IRFs)

While the JAKs and STATs play an important role in IFN signaling, the *I*FN *r*egulatory *f*actor (IRF) family of nine transcription factors (IRF1-9) is also involved in IFN signal transduction and IFN production. In addition to these roles, IRFs are involved in immunological functions and cell growth (Nguyen *et al.*, 1997; Taniguchi *et al.*, 2001). The multiple members of this family all share a 125 amino acid homology at the N-terminal region. This region encodes the DNA binding domain that binds to *I*FN *r*esponse elements (IREs) within promoter regions of ISGs. The more divergent C-terminal serves as the regulatory domain and aids in the classification of the IRFs into three groups. The three groups include activators (IRF1, IRF3, IRF7 and IRF9/ISGF 3γ/p48), repressors (IRF2 and IRF8/ICSBP) and lastly those that can behave as either activators or repressors (IRF2 and IRF4/LSIRF/Pip) (Taniguchi *et al.*, 2001). Generally, IRFs are capable of interacting with other IRF proteins and also with

members of other transcription factor families to modify both IRE binding activities and the formation of transcriptional initiation complexes (Figure 1.7).

The first member of this family to be isolated, IRF1, is undetectable or present at low levels in most cells and is able to be induced by a variety of cytokines (Kroger *et al.*, 2002; Miyamato *et al.*, 1988; Pine *et al.*, 1990). Once induced, IRF1 accumulates in the nucleus and binds to specific promoter regions within IRF1 responsive genes. This binding often occurs in association with other transcription factors such as IRF8 or NFκB (Escalante *et al.*, 1998; Fujii *et al.*, 1999; Thanos & Maniatis, 1992; Ten *et al.*, 1993). IRF1 was originally described as a regulator of IFN production (Harada *et al.*, 1990). However, the generation of the *Irf1*$^{-/-}$ mouse illustrated that IRF1 was not necessary for the induction of the type I IFN genes, as production was unaffected. Rather, these mice showed that IRF1 had an important role in immune function. The *Irf1*$^{-/-}$ mice were immunodeficient, lacking the expression of gene products involved in antigen presentation, Th1 and NK cell responses (Matsuyama *et al.*, 1993; Duncan *et al.*, 1996; White *et al.*, 1996; Hobart *et al.*, 1997; Lohoff *et al.*, 1997; Taki *et al.*, 1997). Another important function of IRF1 is its tumor suppressor roles. Fibroblasts deficient for IRF1 exhibit a failure to induce apoptosis and instead are rapidly transformed by activated oncogenes (Tanaka *et al.*, 1994).

IRF2 was discovered as a repressor of IFN and ISG expression, as it bound to the same promoter elements as IRF1 and also in ISRE regions which the ISGF3 complexes bind (Miyamoto *et al.*, 1988; Harada *et al.*, 1990). It was believed that IRF1 and IRF2 competed for the same binding sites within promoter regions of ISGs and IFNs, so that when IRF2 bound, IRF1 and ISGF3 were inhibited (Palombella & Maniatis, 1992; Taniguchi, 1995). When IRF2 is overexpressed in NIH 3T3 cells, the cells undergo transformation demonstrated by growth in soft agar and as tumours when injected into nude mice, implying that IRF2 possesses oncogenic properties (Harada *et al.*, 1993). The generation of the *Irf2*$^{-/-}$ mouse supported the importance of IRF2 as a regulator of IFN signaling. In the absence of IRF2, responses to type I IFNs are uncontrolled which leads to abnormal $CD8^+$ T cell activation (Hida *et al.*, 2000). These mice also exhibited an inability to respond to IL12 and therefore fail to

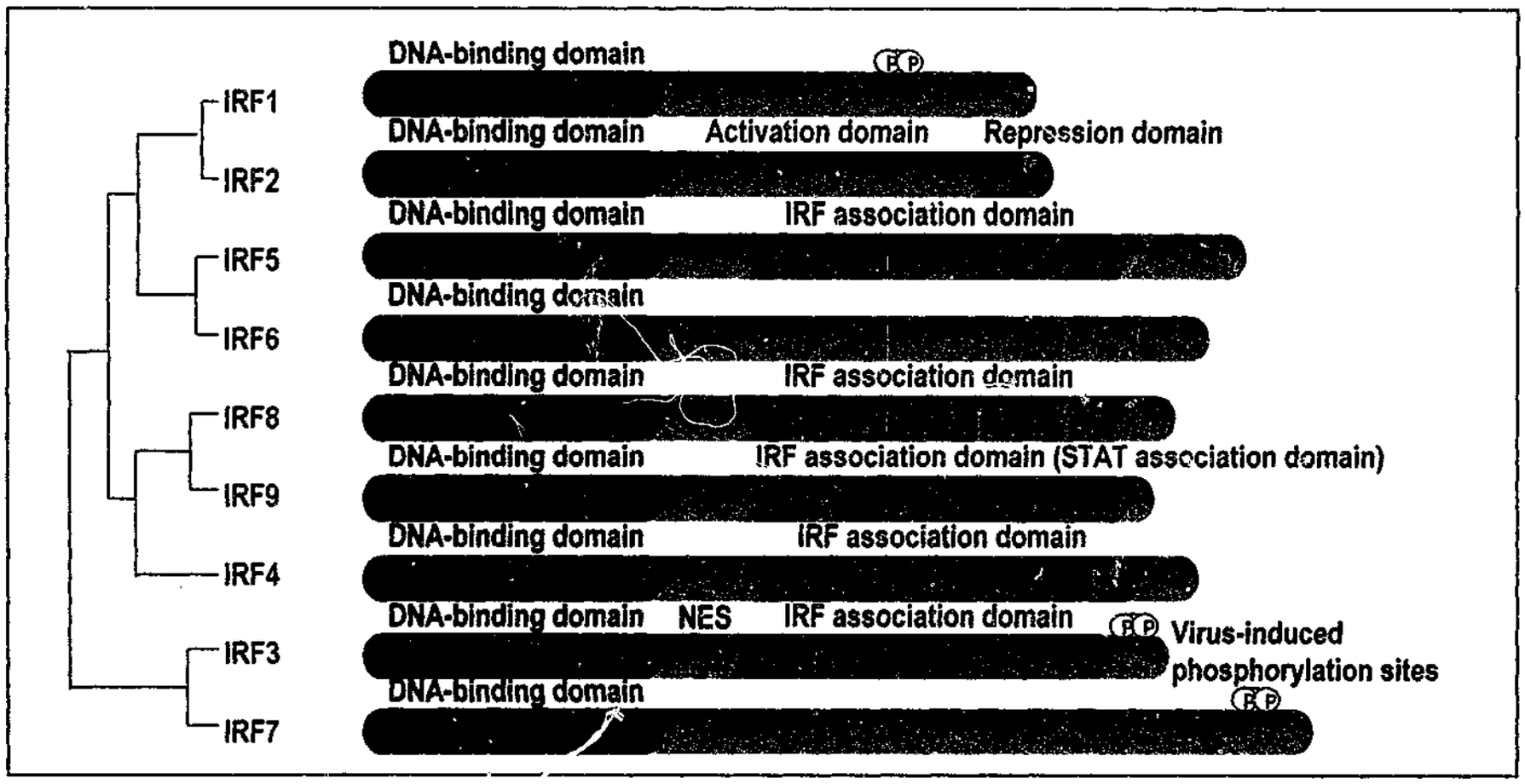


Figure 1.7: IRF Protein Structures and Phylogenetic Relationships.

Members of the IRF family of proteins share a 125 amino acid homology at the N-terminal region encoding the DNA-binding domain. The C-terminal regions encoding the regulatory domains are more divergent. NES: nuclear export signal (Adapted from Taniguchi *et al.*, 2001).

induce Th1 cell differentiation and NK cell development (Lohoff *et al.*, 2000) which made these mice susceptible to viral infection. The *Irf2* deficient mice develop an inflammatory skin disease that is caused by a continual low level of ISGF3 induction, suggesting that IRF2 is required to keep a balance between the beneficial and harmful effects of the type I IFNs (Hida *et al.*, 2000).

IRF9 previously known as p48 or ISGF3γ as it was IFNγ inducible (Bandyopadhyay *et al.*, 1990; Levy *et al.*, 1990; Eilers *et al.*, 1993; Weihua *et al.*, 1997; Wong *et al.*, 1998; Matsumoto *et al.*, 1999), forms a heterotrimeric transcription factor, binding to the STAT1:STAT2 heterodimer through its carboxyl-terminal domain termed as *I*FN *s*timulated *g*ene *f*actor *3* (ISGF3). This transcriptional activation complex is then able to cross the nuclear membrane where IRF9 and STAT1 are able to bind to the DNA in specific regions termed ISREs (Horvath & Darnell, 1996; Martinez-Moczygemba *et al.*, 1997). Mice deficient for *Irf9* fail to survive viral infection, which is not surprising as no ISGF3 dependent genes are able to be induced in response to IFN (Kimura *et al.*, 1996). Human cells which lack *IRF9* are unable to express IRF7, therefore no *IFNA* genes are able to be induced (Sato *et al.*, 1998).

IRF3 was identified through EST database mining for homologs to IRF1 and 2 (Au *et al.*, 1995). Subsequently, IRF3 was found to be present at high levels in the cytoplasm of all cell types in a dormant form. IRF3 is activated downstream of TLR3 recognition of virus and TLR4 recognition of LPS which through the adapter molecule TRIF/TICAM1 activate the kinases IKKε and TBK1 (Fitzgerald *et al.*, 2003). Once IRF3 is activated it plays a very important role in the induction of IFN genes following viral infection, as previously discussed (Marie *et al.*, 1998; Noah *et al.*, 1999; Sato *et al.*, 2000; Yeow *et al.*, 2001; Yoneyama *et al.*, 1998). The closest relative to IRF3 is IRF7, which also plays a significant role in IFN gene induction, albeit in the late amplification phase of gene activation. IRF7 was originally cloned as a factor that bound to a promoter region in the Epstein-Barr virus using a yeast one-hybrid system (Zhang & Pagano, 1997). IRF7, once induced, is present in the cytoplasm as an inactive protein that is also phosphorylated on specific C-terminal residues by TBK1

and/or possibly IKKε kinases (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). IRF7, as previously discussed, has an important role in the regulation of *IFNA/Ifna* genes. Human cells deficient for *IRF7* are unable to induce the expression of these genes, however when cells are reconstituted with functional *IRF7*, viral stimulation of *IFNA* genes occurs (Yeow *et al.*, 2000). Therefore it appears that both IRF3 and IRF7 together are responsible for the induction for the full spectrum of the type I IFN genes in response to viral infection.

The *I*FN *c*onsensus *s*equence *b*inding *p*rotein (ICSBP)/IRF8 was originally identified as a protein that bound to ISREs in the promoter region of MHC class I (Driggers *et al.*, 1990; Weiz *et al.*, 1992). Expression of IRF8 is induced by IFNγ, not IFNα/β and is restricted to myeloid and lymphoid cell lines (Driggers *et al.*, 1990; Nelson *et al.*, 1996). The function of IRF8 is similar to that of IRF2, in that it represses ISG expression (Nelson *et al.*, 1993; Bovolenta *et al.*, 1994). *Irf8*$^{-/-}$ mice have been generated and present with *c*hronic *m*yelogenous *l*eukemia (CML)-like disease and immunodeficiency (Holtschke *et al.*, 1996). Interestingly, patients that suffer from CML have suppressed IRF8 expression (Schmidt *et al.*, 1998). When these patients are administered IFNα, IRF8 expression is restored, suggesting that *IRF8* may act as a tumor suppressor (Schmidt *et al.*, 1998).

The remaining IRF family members, IRF4, 5 and 6, are the least characterised. IRF4 induces immunoglobulin gene expression and is essential for maturation and homeostasis of lymphocytes. Expression of IRF4 is limited to T and B cell lineages and is not induced by either type I or II IFN (Eisenbeis *et al.*, 1995; Matsuyama *et al.*, 1995; Yamagata *et al.*, 1996). *Irf4*$^{-/-}$ mice have been generated and are immunodeficient with defects in both B and T cell proliferation resulting in an imbalance in lymphocyte homeostasis (Mittrucker *et al.*, 1997). Both IRF5 and 6 are structurally related, IRF5 is induced by IFNα/β however induction of IRF6 is unknown (Taniguchi *et al.*, 2001).

1.6 SIGNALING PATHWAYS

Cells within the body utilise signaling pathways to initiate processes within cells to induce expression of specific proteins that can perform particular biological effects. An example of this is the JAK/STAT pathway that is important for relaying messages from various cytokines from the extracellular compartment into cells to change gene expression and thereby generate biological effects.

1.6.1 JAK/STAT Pathway

Signal transduction, as a result of IFN action, is initiated when IFN has bound to the cell surface receptor. The JAK/STAT pathway is the best characterised of the signal transduction pathways through which both the type I and II IFN signal (Darnell *et al.*, 1994).

1.6.1.1 Type I Interferon Signaling via the JAK/STAT Pathway

Both the IFNAR1 and 2 chains lack intrinsic kinase activity, but both chains are associated with JAK kinases; IFNAR1 with TYK2 and IFNAR2 with JAK1 (Stark & Kerr, 1992; Richter *et al.*, 1998; Kotenko *et al.*, 1999). This association enables activation of the receptor complex to proceed upon IFN binding to the receptor chains. The type I IFNs are comprised of multiple proteins (IFNαs and IFNβ) and all can compete for binding to the one receptor, IFNAR (Pestka *et al.*, 1987; Stark *et al.*, 1998). Past research has indicated that certain type I IFNs are able to exercise their specificity by binding to different regions of the IFNAR receptors (e.g. IFNβ) (Lewerenz *et al.*, 1998). The receptor, after the ligand has bound, takes on different conformations with the different IFN subtypes (Cleary *et al.*,1994; Cook *et al.*, 1996; Cutrone *et al.*, 1997). For instance a study completed by Domanski *et al.* (1998) demonstrated that IFNα2 and IFNβ interact with different regions of the IFNAR subunits to generate an antiviral response. In order for specific reponses of IFN to occur, the various subtypes would need to bind the IFNAR receptor with some degree of specificity.

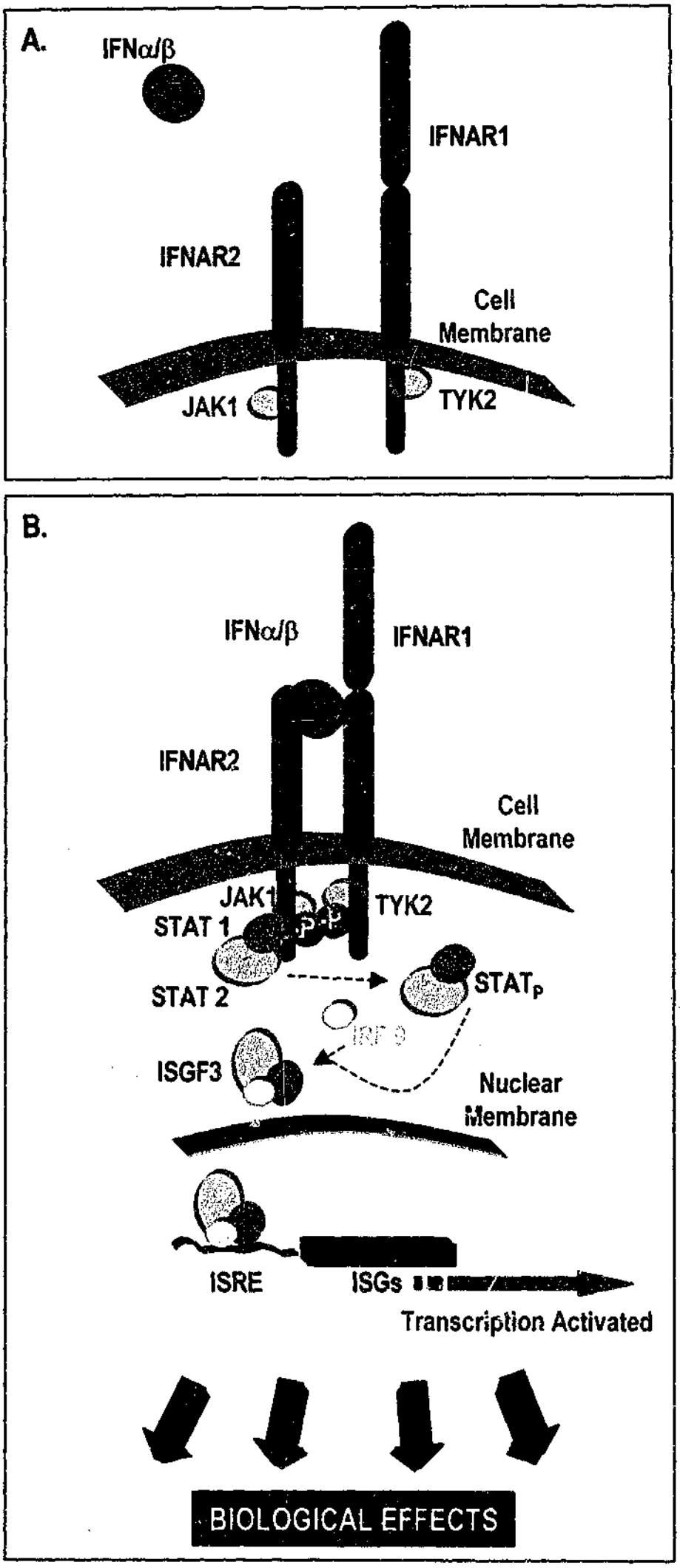
Understanding these mechanisms of specificity is of interest to fully comprehend the extent of type I IFN signaling.

IFNAR2 is believed to be the main ligand binding chain of the type I IFN receptor, while IFNAR1 also contributes. TYK2 is brought into the activated receptor complex through its preassociation with IFNAR1 and is thought to add stability of IFNAR1 in the membrane and as a result increases binding affinity (Velasquez *et al.*, 1995). Ligand binding results in the receptor associated JAKs, TYK2 and JAK1 to become phosphorylated by a trans-phosphorylation mechanism, phosphorylating each other on tyrosine residues (Barbieri *et al.*, 1994; Gauzzi *et al.*, 1996; Velazquez *et al.*, 1992). The cytoplasmic regions of the two chains of the receptor then become phosphorylated, Tyr^{466} on IFNAR1 (Yan *et al.*, 1996) and Tyr^{337} or Tyr^{512} on IFNAR2c (Wagner *et al.*, 2002) thus initiating the signal transduction cascade. Once the IFNAR2 chain is phosphorylated it is able to recruit latent STAT2 molecules, which become phosphorylated and subsequently bind to STAT1 molecules. This allows STAT1 to be phoshorylated by the receptor associated JAKs. Once both STAT1 (STAT 1α and β) and STAT2 are phophorylated they heterodimerise and are released from the receptor complex due to their lower affinity for the receptor. Subsequently, this heterodimer binds IRF9 to form the *i*nterferon *s*timulated *g*ene *f*actor 3 (ISGF3) (Schindler & Darnell, 1995; Bluyssen *et al.*, 1996). This multiprotein signaling complex then translocates to the nucleus where it binds to *i*nterferon *s*timulated *r*esponse *e*lements (ISREs) within the promoter regions of type I *i*nterferon *s*timulated *g*enes (ISGs) through the consensus binding site AGTTTCNNTTTCNPy (Darnell, 1997; Fu *et al.*, 1992; Stark *et al.*, 1998) (Figure 1.8). The ISRE is largely responsible for IFN regulated gene expression. However, STAT1 homodimers can be initiated from type I IFN induction. These homodimers then translocate to the nucleus and bind to *g*amma *a*ctivated *s*equence (GAS) elements within promoter regions of ISGs (Owczarek *et al.*, 1997).

Figure 1.8: Type I IFN Signaling via the JAK/STAT Pathway.

A. In the absence of type I IFN ligand, JAK1 and TYK2 associate with IFNAR2 and IFNAR1 respectively (Stark & Kerr, 1992).

B. Ligand binding to the IFNAR2 recruits IFNAR1 with TYK2 to the receptor complex, the two JAK family members then trans-phosphorylate one another as well as the cytoplasmic domains of the two receptor subunits. The activated phosphorylated receptor subunit forms a docking site for STAT2 which binds via SH2 domain after which STAT1 is recruited and subsequently phosphorylated. Heterodimerisation of the STAT1 and 2 phosphorylated proteins occurs and dissociates from the receptor. IRF9 then binds the STAT1 and 2 heterodimer, forming a heterotrimeric transcription factor, ISGF3 which translocates to the nucleus where IRF9 and STAT1 bind to ISREs on the DNA within promoter regions of ISGs thus initiating transcription. Induced ISG products ultimately result in biological effects.


A.
IFNα/β
IFNAR1
IFNAR2
Cell
Membrane
JAK1
TYK2
B.
IFNα/β
IFNAR1
IFNAR2
Cell
Membrane
JAK1
TYK2
STAT 1
STAT 2
STAT$_P$
IRF 9
ISGF3
Nuclear
Membrane
ISRE
ISGs
Transcription Activated
BIOLOGICAL EFFECTS

1.6.1.2 Type II Interferon Signaling

In contrast to IFNα/β, IFNγ binds as a homodimer to its receptor (Bach *et al.*, 1997) which consists of two subunits, IFNGR1 and IFNGR2. In order to initiate a signaling cascade induced by IFNγ, the ligand must bind the IFNGR1 subunits that are associated with JAK1 kinases. Then, in order for a fully assembled receptor complex to be formed and hence optimal signal transduction to occur, the recruitment of the second chains of the receptor, IFNGR2 are required, as well as the associated JAK2 kinases (Kotenko *et al.*, 1995). Once the receptor complex has formed the associated JAKs are activated, as described above for the type I IFNs in a trans-phosphorylation mechanism. This enables latent STAT1 molecules to be recruited to the IFNGR1 chains where they bind to the docking sites formed on the phosphorylated IFNGR1 subunit (phosphotyrosine motif Y^{457}) and are subsequently phosphorylated themselves on tyrosine^{701} (Bach *et al.*, 1997; Greenlund *et al.*, 1995). The IFNGR2 chains do not contain STAT recruitment or binding sites, and thus do not participate in the STAT recruitment process. Rather their role is earlier in the pathway with the recruitment of JAK2 to the receptor complex which is required for the signal cascade to occur as demonstrated by *Ifngr2*$^{-/-}$ studies (Briscoe *et al.*, 1996). *Ifngr2*$^{-/-}$ mice are unable to respond to IFNγ due to the lack of the recruitment of JAK2 to the receptor complex by IFNGR2. Both JAK1 and JAK2 IFNγ induced phosphoryation was absent in *Ifngr2*$^{-/-}$ mice suggesting that IFNGR2 along with preassociated JAK2 recruitment was required to initiate IFNγ signaling events (Kontenko *et al.*, 1995; Briscoe *et al.*, 1996; Lu *et al.*, 1998). Subsequently, the phosphorylated STAT1 molecules homodimerise via their SH2 domains and translocate to the nucleus. Once in the nucleus the STAT1 homodimers bind GAS elements within the promoter regions of ISGs to induce gene expression (Schindler & Darnell 1995) (Figure 1.9).

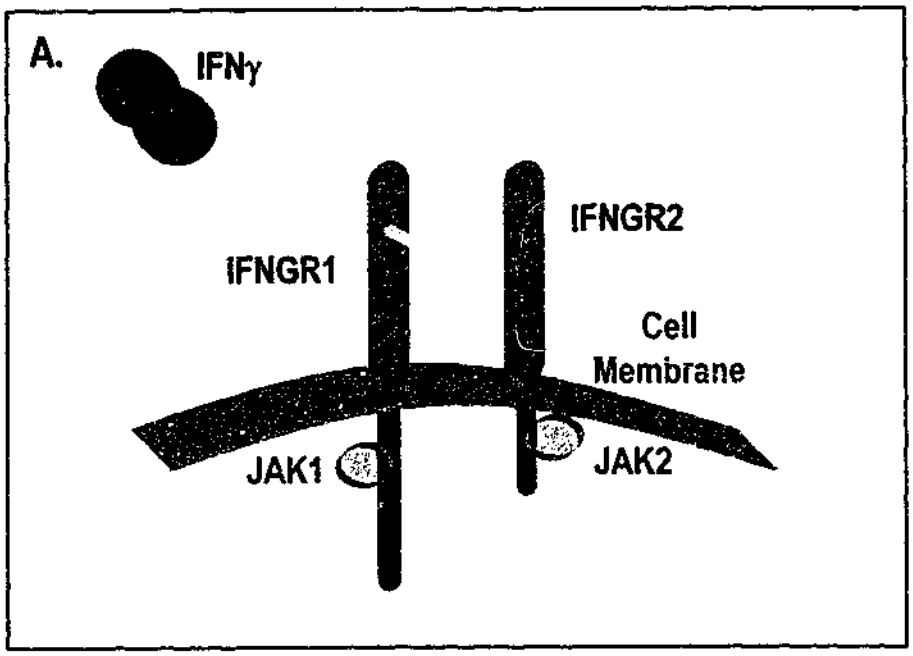
1.6.2 Non JAK/STAT Signaling Pathways

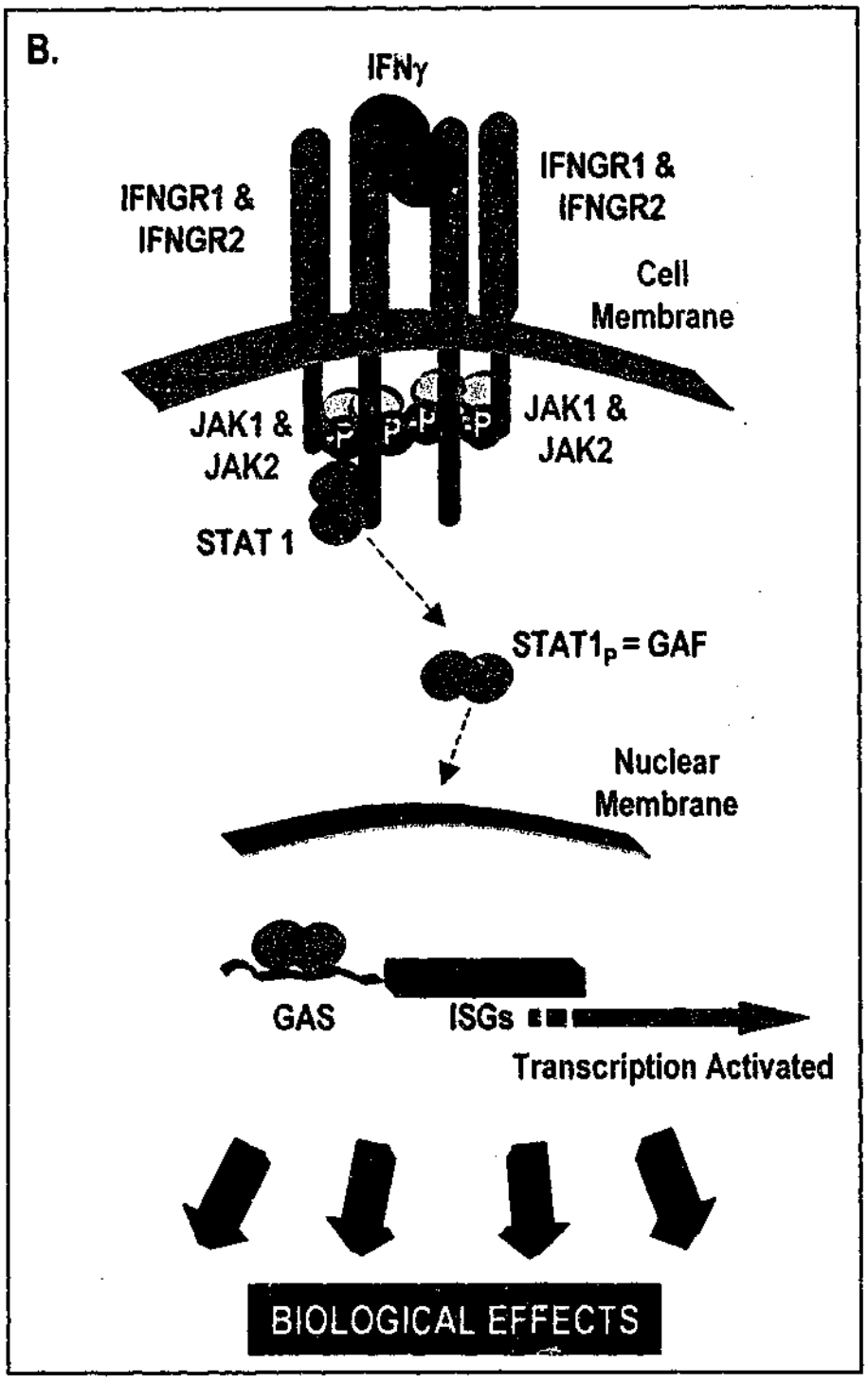
It has only been recently demonstrated that the JAK/STAT pathway is not the only pathway that will activate transcription of IFN induced genes.

Figure 1.9: Type II IFN Signaling via the JAK/STAT Pathway.

A. In the absence of type II IFN ligand, JAK1 and JAK2 associate with IFNGR1 and IFNGR2 respectively. The two subunits of the receptor are not associated.

B. Ligand binding to two IFNGR1 subunits recruits 2 IFNGR2 with JAK2 to the receptor complex, the JAK family members then trans-phosphorylate one another as well as the cytoplasmic domains of the four receptor subunits. The activated phosphorylated receptor subunit forms a docking site for STAT1 which binds via SH2 domain after which another STAT1 is recruited and subsequently phosphorylated. Homodimerisation of the phosphorylated STAT1 proteins occurs and dissociates from the receptor as a *g*amma *a*ctivated *f*actor (GAF). GAF then translocates to the nucleus where STAT1 molecules bind to *g*amma *a*ctivated *s*equence (GAS) of DNA within promoter regions of ISGs and thus initiating transcription. ISG products ultimately result in biological effects.


A.
IFNγ
IFNGR2
IFNGR1
Cell
Membrane
JAK1
JAK2


B.
IFNγ
IFNGR1 &
IFNGR2
IFNGR1 &
IFNGR2
Cell
Membrane
JAK1 &
JAK2
JAK1 &
JAK2
STAT 1
STAT1p = GAF
Nuclear
Membrane
GAS
ISGs
Transcription Activated
BIOLOGICAL EFFECTS

1.6.2.1 Type I IFN Signaling Specific to T cells

An IFNα growth inhibitory signal in lymphocytes requires the expression and association to IFNAR of phosphatase CD45 and tyrosine kinases LCK and ZAP70, signaling proteins that have best been characterised for their role in T cell receptor signaling (Petricoin *et al.*, 1997). This was demonstrated by a series of immunoprecipitation/Western blotting experiments where the activation, (phosphorylation) of CD45, LCK, ZAP70 and presumably the association with the IFNAR complex is a requirement for the antiproliferative actions of IFNα in T cell lines. In cells that were deficient in CD45, LCK or ZAP70, IFN failed to induce an antiproliferative response, even though the ISGF3 complexes still formed, further supporting the requirement for these proteins (Petricoin *et al.*, 1997, Penninger *et al.*, 2001). This data suggests that JAK/STAT activation is not sufficient for an antiproliferative effect in these cells. It is believed that IFNα binds to the IFNAR complex on the T cell, bringing not only the two chains of the receptor together, which in a classic situation would be sufficient to induce the JAK/STAT response, but also the cell surface CD45. The association of CD45 with the type I IFN receptor then recruits the LCK followed by the ZAP70 components of the T cell to produce a cascade resulting in antiproliferative activity (Figure 1.10) (Petricoin *et al.*, 1997). The role of the T cell components that Petricoin *et al.* (1997) elucidated in the action of IFNα in T cells suggests that in other cell types where IFNα results in growth inhibition, other pathways must be considered as being potentially important in the antiproliferative effects of this cytokine.

1.6.2.2 Type I IFN in Insulin Receptor Substrate and Phosphatidylinositol 3-Kinase Pathways

Activation of the IFNAR complex can result in the engagement of multiple proteins including STAT1 and 2 as previously discussed as well as STAT3, *i*nsuin *r*eceptor *s*ubstrate 1 (IRS1) and IRS2 (Uddin *et al.*, 1995, 1997, 2000b; Platanias *et al.*, 1996). The IFNAR1 subunit can directly bind to the SH2 domain of STAT3 on phosphorylated residues Tyr^{527} and Tyr^{538} after IFN stimulation (Yang *et al.*, 1996; Pfeffer *et al.*, 1997). Subsequently, STAT3 is

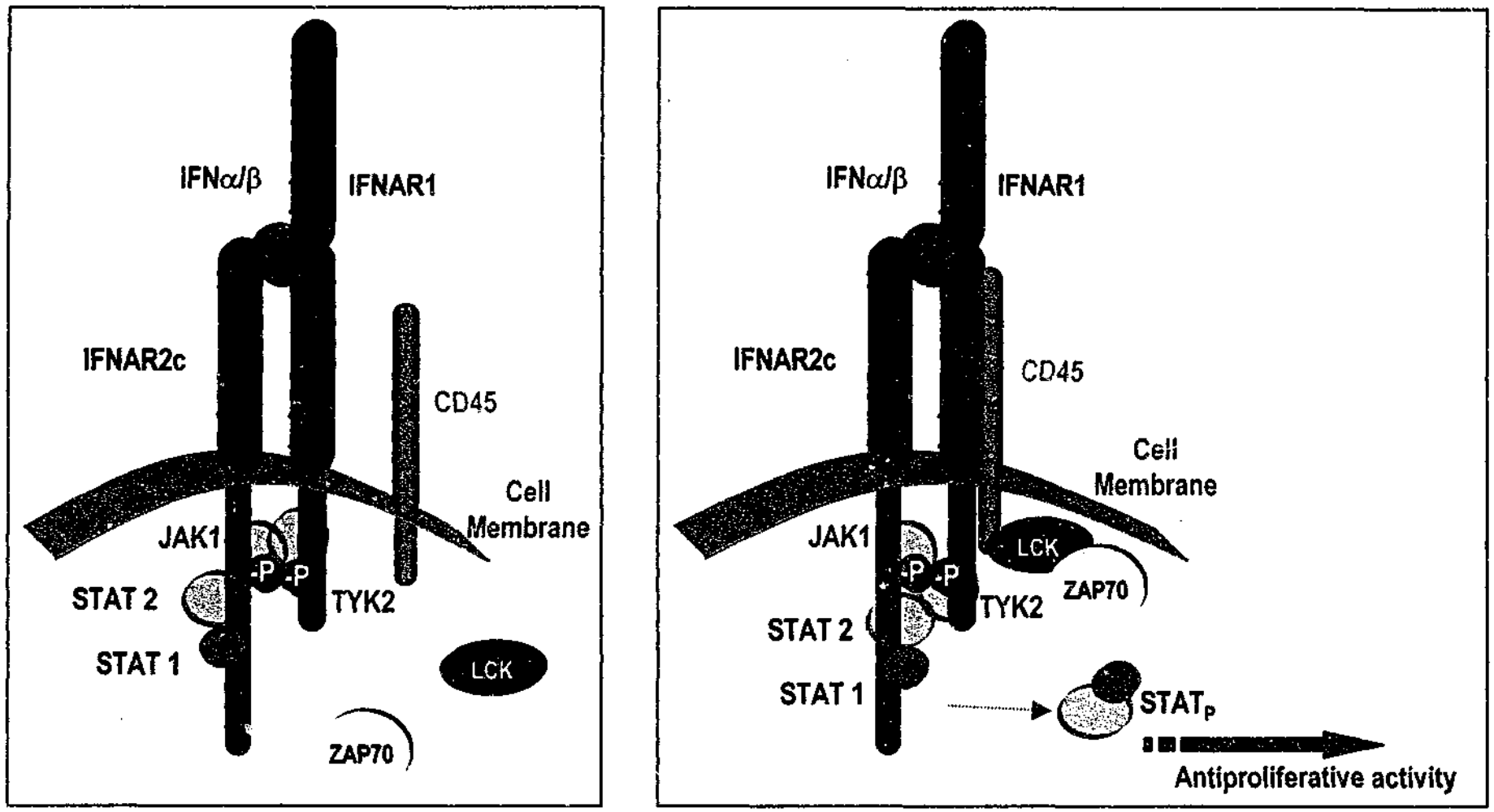


Figure 1.10: A Representation of the T cell Signaling Components working in conjunction with the JAK/STAT pathway (Petricoin *et al.*, 1997).

It has been proposed that T cell signaling components CD45, Lck and ZAP70, associate with the type I IFN receptor complex after IFN has bound in order to elicit an antiproliferative response.

phosphorylated at residues Tyr^{656} and Tyr^{705} which can bind the regulatory p85 subunit of *p*hosphat*i*dylinositol 3-*k*inase (PI3K) which is tyrosine phosphorylated thus acting as an adapter to couple PI3K to IFNAR1 (Pfeffer *et al.*, 1997). This adapter function of STAT3 between IFNα/β signaling and the PI3K pathway has been demonstrated only in Daudi cells.

In other haematopoietic cells, the adapter protein between IFNAR1 and PI3K was IRS1 and 2. IRS1 is the principle substrate of the insulin receptor of non-haematopoietic cells, while IRS2 is the major substrate protein found in haematopoietic cells which lack IRS1 expression (Sun *et al.*, 1995; Platanias *et al.*, 1996). IFNα/β treatment can induce the tyrosine phosphorylation of IRS1 and IRS2 which in turn activates the p85 subunit of the PI3K by serine phosphorylation (Uddin *et al.*, 1995; Uddin *et al.*, 1997; Platanias *et al.*, 1996). In response to type I IFNs, experiments demonstrate that the major signaling pathway that activated PI3K was the IRS pathway (Uddin *et al.*, 2000b; Ruuth *et al.*, 2001). Interestingly this action can occur in the absence of IFNAR being phosphorylated on tyrosine residues (Uddin *et al.*, 2000b). The regulatory subunit of PI3K contains an SH2 domain, which is capable of binding to phosphorylated IRS1 to activate PI3K (Backer *et al.*, 1992; Shoelson *et al.*, 1993; Sun *et al.*, 1993). This pathway has been shown to be functionally important as inhibition of apoptosis in neutrophils and T cells has been shown to be dependant on the activation of PI3K by type I IFNs (Wang *et al.*, 2003). This data indicates that there are many different pathways that utilise the same factors but result in different biological effects.

Current evidence suggests that IFNα/β activation of the type I IFN receptor is able to activate numerous factors such as STAT1, STAT2, STAT3, CD45, IRS1 and IRS2 of which two examples have been discussed above (David *et al.*, 1995; Pfeffer *et al.*, 1997; Platanias *et al.*, 1996; Petricoin *et al.*, 1997). By having the potential to activate numerous factors involved in signaling pathways other than the JAK/STAT, demonstrates how the type I IFNs can diversify their biological outcomes.

1.6.3 Cross Talk between Type I and II Interferon Signaling

It is becoming increasingly apparent that signaling pathways rarely act in isolation. Two pathways that appear to cross talk/overlap are the type I and II IFN pathways. This signaling cross talk provides a molecular mechanism that could account for overlapping biological effects elicited by both IFNα/β and IFNγ. Two examples of similarity between the IFNs are the activation of ISGF3 and the generation of STAT1 homodimers. ISGF3 formation has been demonstrated to be critical for IFNγ induced antiviral responses (Matsumoto *et al.*, 1999; Kimura *et al.*, 1996). However in *Ifnar1*$^{-/-}$ primary embryo fibroblasts, induction of ISGF3 was undetected and an impaired antiviral effect in response to IFNγ was exhibited (Takaoka *et al.*, 2000). This contradicts earlier work that found IFNγ could stimulate the activation of ISGF3 in the absence of IFNAR1 in fibroblasts (Owczarek *et al.*, 1997; Matsumoto *et al.*, 1999). Work by Takaoka *et al.* (2000) demonstrated by expressing mutant forms of IFNAR1 in *Ifnar1*$^{-/-}$ cells, that the antiviral response exhibited in response to IFNγ only occurred with the expression of wild-type IFNAR1.

A characteristic of viral infection is elevated levels of type I IFN, which has classically been thought to function as the body's first line of defence. In contrast other cytokines, including IFNγ, become more important as the infection progresses. However both the type I and II IFNs are able to induce similar immune activity. IFNγ is able to activate T cells to target infected cells for destruction (Frucht *et al.*, 2001), however type I IFNs also induce multiple immunoregulatory effects on NK and T cells during viral infection (Belardelli & Gresser, 1996; Nguyen *et al.*, 2000). More specifically the type I IFNs can induce NK cell proliferation and cytotoxicity. Type I IFNs also promote the survival of memory T cells (Nguyen *et al.*, 2000). Therefore what was once thought a type II IFN function only, becomes the action of both types of IFN.

A series of studies conducted by different groups has added to the knowledge of overlap between the type I and II IFN signaling pathways. One set of results investigated the IFNγ priming effect in IFN resistant melanoma cells to reveal

that by pre-treating these cells with IFNγ and then subsequently treating with type I IFN, levels of ISGF3 were elevated resulting in an augmented response to type I IFNs (Levy *et al.*, 1990; Wong *et al.*, 1998). This was due to an increase in both STAT1 and IRF9 subunits which could be attributed to IFNγ, as IRF9 was originally termed ISGF3γ, an IFNγ inducible protein (Bandyopadhyay *et al.*, 1990; Levy *et al.*, 1990; Weihua *et al.*, 1997; Matsumoto *et al.*, 1999; Wong *et al.*, 1998). Another study examined the level of IFNAR2 on the cell surface and found that cells treated with IFNγ increased the expression levels of IFNAR2 on the cell surface again increasing the degree of the type I IFN response (Hardy *et al.*, 2002). A third group of studies demonstrated that an association between the IFNAR1 subunit of the type I IFN receptor complex and the IFNGR2 subunit of the type II IFN complex existed to facilitate cross talk. This was demonstrated from immunoprecipitation experiments revealing that IFNAR1 co-precipitated with IFNGR2 before any stimulation (Takaoka *et al.*, 2000). It was concluded from these studies that the tyrosine residues present on the cytoplasmic domain of the IFNAR1 chain were critical for the cross talk to occur, in that they provided the docking site for STAT1 and 2 transcription factors after the stimulation of IFNGR. Interestingly this association was diminished in cells lacking IFNβ, which is highly suggestive that IFNγ signaling through IFNAR1 is facilitated through a constitutive subthreshold/basal level of type I IFN signaling (Takaoka *et al.*, 2000). Together all of these studies have demonstrated that components of both the type I and type II IFN signaling pathways are able to impact on the efficient signaling of either pathway.

Interestingly, to produce efficient activation of the STAT factors in the IL6 pathway, subthreshold/basal levels of IFNα/β are also required in a mechanism postulated to be similar to that with the IFNGR. It was shown that in the absence of type I IFN, the activation levels of STAT1 and 3 are reduced in response to IL6 stimulation (Mitani *et al.*, 2001). Therefore it seems that the type I IFNs are required to be produced in low amounts in order to keep certain pathways (IL6 and IFNγ) primed ready for activation, similarly IFNγ plays the same role for type I IFN responses (Takaoka *et al.*, 2000; Mitani *et al.*, 2001).

Therefore, type I IFN signaling may be an important part of other cytokine signals.

1.7 INTERFERON STIMULATED GENES

In IFN responsive cells, the biological actions (antiviral, antiproliferative and immunoregulatory) of IFNs are elicited by a set of genes known as *i*nterferon *s*timulated *g*enes (ISGs), many of which are activated by IFN via the JAK/STAT pathway (Kumar *et al.*, 1994). It is important to note that ISGs can also be induced without the formation of ISGF3. Instead, they can be induced by factors termed as *ds*RNA *a*ctivated *f*actors (DRAFs) 1 and 2 which are complexes of IRF3 and CBP/p300 factors (Daly & Reich, 1993). There has been an exponential growth in the number of identified ISGs since the introduction of gene microarray technology. Within a few experiments the list has increased dramatically from around 50 to well over 1000 (de Veer *et al.*, 2001). Gene microarray experiments have been able to clarify the expression profiles of known ISGs and elucidate novel genes. The results from experiments carried out by de Veer *et al.* (2001) have generated gene lists that are IFNα, IFNβ and IFNγ specific. This data together with the continuing use of microarray technology will enable further functions and the primary effector genes of both type I and II IFN to be elucidated. Table 1.2 lists some of these ISGs of interest to this project along with their main biological effects.

1.7.1 2′-5′ Oligoadenylate Synthetase (2′-5′ OAS)

The 2'-5' oligoadenylate synthetases comprise a family of well-characterised type I IFN induced antiviral proteins that once induced are activated by dsRNA (Sen & Lengyel, 1992; Rebouillat & Hovanessian, 1999). The function of these enzymes is to produce oligoadenylate chains in a 2'-5' linkage that activates the RNaseL protein which degrades both viral and cellular RNA resulting in the suppression of protein synthesis and hence viral growth (Dong & Silverman, 1995; Ronni *et al.*, 1997). Transfection of cells with 2'-5' OAS induces an antiviral state independent of IFN demonstrating the direct antiviral activity of this ISG. The importance of RNaseL has been shown by the generation of

Table 1.2: Interferon Inducible Genes and their Biological Relevance.

(Ucar *et al.*, 1995; D'Cunha *et al.*, 1996; Min *etal.*, 1996; Oritani *et al.*, 2001; Decker *et al.*, 2002)

Gene	Biological Effect	Function	Inducing IFN
2'-5' Oligoadenylate Synthetase	Antiviral	Degrades viral mRNA	Type I
RNaseL	Antiviral	Degrades both viral and cellular RNA after viral infection, resulting in apoptosis and virus inhibition	Type I
PKR kinase	Antiviral	Important for the induction of antiviral state; inhibits protein synthesis	Type I and II
Mx proteins	Antiviral	Inhibits viral replication	Type I
MHCI	Immunoregulatory	Antigen processing and presentation; promotes cytotoxicity	Type I
IP10	Immunoregulatory	Secreted protein capable of regulating migration, activation and maturation of leukocytes	Type II
iNOS	Antiviral Immunoregulatory	Protective role against bacterial and viral infections	Type I and II
SOCS1	Negative Regulation	Inhibits JAK/STAT signaling pathways by binding to	Type I and II
SOCS3	Negative Regulation	Inhibits JAK/STAT signaling pathways by binding to phosphorylated cytokine receptors	Type I and II

RNaseL$^{-/-}$ mice which results in a deficiency in IFN induced antiviral activity (Zhou *et al.*, 1997). These mice also exhibit enlarged thymi with thymocytes that are resistant to apoptotic stimuli and are more susceptible to virus due to the lack of IFN signaling that would normally result in apoptosis via the activation of the 2'-5' OAS enzyme (Zhou *et al.*, 1997). (Figure 1.11).

1.7.2 Protein Kinase dsRNA Activated Protein Kinase (PKR)

PKR is a dsRNA dependent serine/threonine protein kinase that is able to bind as a dimer directly to dsRNA (Williams, 1997; Patel *et al.*, 1995). Both before and after IFNα stimulation, PKR is localised to the cytoplasm of cells in association with ribosomes (Pestka *et al.*, 1987; Samuel, 1993; Thomis *et al.*, 1992). PKR is autophosphorylated by either intra- or intermolecular mechanisms after dsRNA binding and also then phosphorylates other protein substrates resulting in inhibition of protein synthesis (Hershey, 1991; Samuel, 1993; Sen, 2001). These other proteins include *e*ukaryotic translation *i*nitiation *f*actor (eIF-2α) (Hershey, 1991), the TAT proteins of HIV (McMillian *et al.*, 1995), NFAT protein and MPP4 (Patel *et al.*, 1999). *In vitro* studies have demonstrated PKR has intrinsic antiviral, antiproliferative and tumor suppressor functions (Clemens & Elia, 1997; Gale & Katze, 1998; Kaufman, 2000; Katze, 1995). Mice deficient for *Pkr* are physically normal, however demonstrate a modest susceptibility to viral infection most likely due to decreased IFNα antiviral signaling (Stojdl *et al.*, 2000). These mice have unimpaired type I IFN signaling, show no signs of tumour formation implying together with the *in vitro* data that the PKR pathway can be compensated to a degree by other antiviral pathways, most likely type I IFN induced (Berlanga *et al.*, 1999).

1.7.3 MX1

The MX family of GTPase proteins, comprised of MXA/MX1 and MXB/MX2 were discovered on the basis of their antiviral functions in response to influenza virus in a strain of influenza resistant mice (Staeheli & Sutcliffe, 1988;

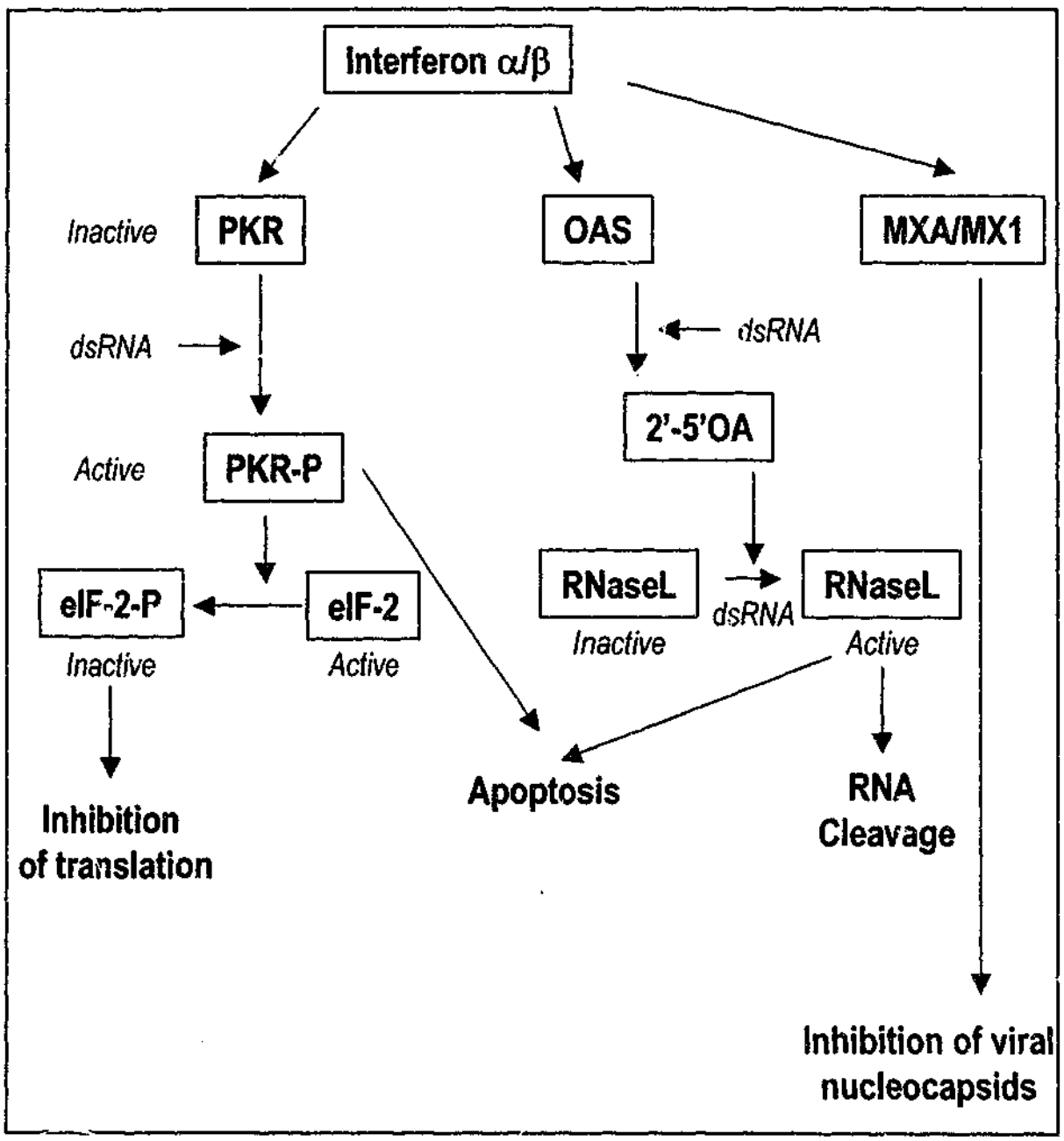


Figure 1.11: Virus Induced Gene Function.

IFNα/β induces the production of PKR, 2'-5' OAS and MX1 in response to virus. These proteins are then able to combat viral infection in both individual and overlapping ways (Adapted from Garcia-Sastre, 2002).

Horisberger & Gunst, 1991; Horisberger, 1995). Therefore mice that were *Mx1*$^{-/-}$ were not resistant to the effects of influenza. The expression of the *Mx* genes is regulated by type I IFNs (Von Wussow *et al.*, 1990; Pavlovic *et al.*, 1993), however they can also be induced directly by virus (Daly & Reich, 1993). This means that during a viral infection, *Mx* gene products are induced in order to carry out antiviral functions within the cell of infection (Arnheiter & Meier, 1990; Horisberger, 1995). The IFN produced resulting from the infection will act on neighbouring cells to induce the expression of *Mx* in these cells rendering them resistant to viral infection (Bazhan & Belova, 1999). IRF1 has been implicated in the induction of *Mx*, as *Irf1* is increased 2-fold in response to influenza (Ronni *et al.*, 1995), which is believed to then translate into functional IRF1 and initiate *Mx1* gene expression (Ronni *et al.*, 1995). It has also been suggested that IFN is able to synergize with the effects of virus to induce higher levels of *Mx1* (Goetschy *et al.*, 1989). The function of *Mx1* has been investigated in mouse cells, and results have illustrated that IFN induced *Mx1* is able to interfere with an early step in viral replication by inhibiting the elongation of viral mRNAs (Pavlovic *et al.*, 1992; Landis *et al.*, 1998) (Figure 1.11). Virally induced *Mx1* clearly interferes with a later step in viral replication, as the induction of MX1 by virus occurs in an already virally infected cell (Bazhan & Belova, 1999).

1.7.4 Suppressors of Cytokine Signaling (SOCS)

The SOCS proteins elicit a negative regulatory effect on signal transduction pathways by acting upon JAKs associated with cytokine receptors, STAT molecules or in some cases directly on the cytokine receptors themselves (Nicholson *et al.*, 1999; Yasukawa *et al.*, 1999). It has become evident that this large family of proteins can be induced via several cytokines that predominantly act by JAK/STAT signal transduction. Once induced, these proteins can then negatively regulate the JAK/STAT pathway in different ways including both binding to the activation loops of JAKs to inhibit phosphorylation and competing with STATs to bind STAT docking sites of activated receptors. Another mechanism that the SOCS proteins can elicit negative regulation is targeting all

bound proteins for proteosomal degradation (Yasukawa *et al.*, 1999; Nicholson *et al.*, 1999).

1.8 NEGATIVE REGULATION

Cytokines are produced by cells in response to various stimuli and have functions beneficial to host defense but may also be potentially lethal. It is therefore important for the body to be able to finely balance these responses. To facilitate such a balance, negative regulation exists to limit the duration and magnitude of cytokine actions. Negative regulation of signaling pathways exists at several levels. Soluble receptors are able to act in a regulatory capacity at the extracellular level, whereas receptor down regulation, involving internalisation and subsequent degradation occurs at the membrane level (Kim & Maniatis, 1996; Strous *et al.*, 1996). Dephosphorylation of signaling molecules via cytoplasmic and receptor bound protein tyrosine phosphatases (e.g. SHP1 and CD45), inhibition of STAT activity by *p*rotein *i*nhibitors of *a*ctivated *S*TAT (PIAS) family (Chung *et al.*, 1997; Neel, 1997; Irie-Sasaki *et al.*, 2001) and the *s*uppressors *o*f *c*ytokine *s*ignaling (SOCS) family of proteins occurs at the cytoplasmic signaling level. Regulation is also able to occur on a transcriptional level via the activation of repressors such as IRF2 that are able to bind to promoter regions of genes to inhibit their transcription (Palombella & Maniatis, 1992; Taniguchi *et al.*, 1995) (Figure 1.12). There are numerous mechanisms by which negative regulation can occur in terms of IFN signaling, of which a few examples will be further discussed.

1.8.1 Protein Tyrosine Phosphatases

It is commonly thought that protein tyrosine kinases are involved in the initiation of positive signaling, while *p*rotein *t*yrosine *p*hosphatases (PTPs) are important in the inhibition of tyrosine phosphorylation dependent signaling by regulating phosphorylation of tyrosine kinase substrates. PTPs form a large family of enzymes that are structurally diverse and elicit a variety of effects in the regulation of cellular signaling events and therefore play an integral role in the balance of homeostasis (Tonks & Neel, 2001). PTPs are classified into 3 broad

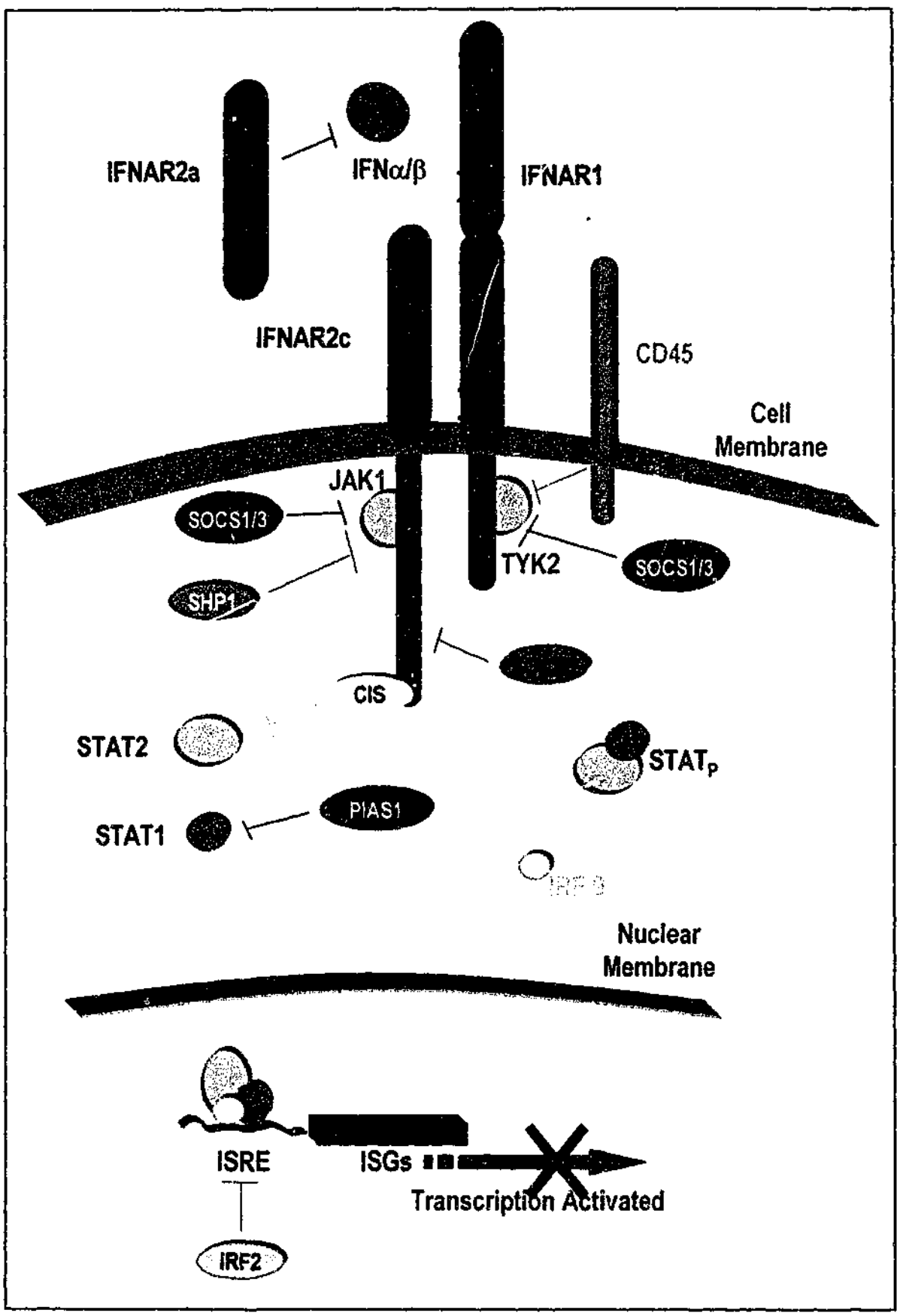


Figure 1.12: Negative Regulators of Type I IFN Signaling.

Numerous proteins are able to have a negative effect on different components of the JAK/STAT pathway induced by type I IFNs. Factors discussed include phosphatases SHP1 and CD45, PIAS, SOCS1, SOCS3, CIS and IRF2.

groups which share a conserved catalytic domain of approximately 240 amino acids which when activated, dephosphorylates the phosphate group in target substrates of particular signaling pathways (Yuvaniyama *et al.*, 1996). These groups are classical PTPs that exist as transmembrane *r*eceptors *PTP*s (RPTPs) and non-transmembrane receptors PTPs, *d*ual *s*pecificity *p*hosphatases (DSPs) and *l*ow *m*olecular weight *p*hosphatases (LMPs) (Neel & Tonks, 1997).

1.8.1.1 SH2 Containing Protein Tyrosine Phosphatase 1 (SHP1)

The tyrosine phosphatase SHP1 is an example of a classic PTP that contains a SH2 domain and is expressed primarily in haematopoietic cells. Studies of the "motheaten" mice which are *Shp1*$^{-/-}$, demonstrated the importance of this phosphatase in regulating haematopoetic development. These mice suffer from multiple haematopoietic abnormalities including autoimmunity and macrophage hyperactivation which are indicative of a malfunction in negative regulation (Shultz *et al.*, 1993; Tsui *et al.*, 1993; Shultz *et al.*, 1997). In *Shp1*$^{-/-}$ macrophages stimulated with IFNα, an increase in the amplitude of JAK1 phosphorylation is observed (David *et al.*, 1995b). Therefore, the recruitment of SHP1 to the receptor complex results in the dephosphorylation of the receptor associated tyrosine kinases, in this case JAK1 (Haque *et al.*, 1998; Migone *et al.*, 1998; Weiss & Schlessinger, 1998). Interestingly in the same experiment with IFNα treated *Shp1*$^{-/-}$ macrophages, the levels of phosphorylated TYK2 were no different from the wild-type macrophages implying that there was some specificity in the target substrates of SHP1 (David *et al.*, 1995b). Other receptor complexes sensitive to SHP1 include the IL3 receptor, *e*rythro*p*oietin (Epo) receptor, steel factor and *c*olony *s*timulating *f*actor *1* (CSF1) (Chen *et al.*, 1996; David *et al.*, 1995b; Klingmuller *et al.*, 1995; Paulson *et al.*, 1996; Yi *et al.*, 1993).

1.8.1.2 CD45

CD45 was discovered as the first transmembrane PTPase (Charbonneau *et al.*, 1988). The structure of CD45 differs from SHP1 in that it is a transmembrane receptor and contains two PTPase domains with one lacking enzyme activity. It has been found through the use of *Cd45*$^{-/-}$ cells that CD45 is a critical positive regulator of the T cell receptor and the B cell receptor in the activation and development of lymphocytes (Kishihara *et al.*, 1993; Byth *et al.*, 1996). CD45 has also been demonstrated to be a JAK phosphatase in cells of haematopoietic lineage in response to a variety of cytokines including IL3, IL4, EPO and IFNα (Irie-Sasaki *et al.*, 2001; Penninger *et al.*, 2001). More specifically CD45 has been shown to be able to dephosphorylate and hence inactivate all four JAKs *in vitro* (Irie-Sasaki *et al.*, 2001). It has been suggested that the second of the two PTPase domains of CD45 physically associate with the JAKs second non-catalytic kinase domain after which CD45 is able to dephosphorylate critical tyrosine residues rendering the JAKs inactive (Irie-Sasaki *et al.*, 2001). The importance of CD45 in IFNα antiviral activity has been demonstrated in *Cd45*$^{-/-}$ mice which are more resistant to lethal Coxsackie viral infection (Irie-Sasaki *et al.*, 2001). Interestingly CD45 has the ability to function in a positive capacity in T cell antiproliferative activities in response to IFNα/β (Petricoin *et al.*, 1997) as previously discussed.

1.8.2 Protein Inhibitors of Activated STATs (PIAS)

Activated STAT molecules are another target for negative regulation of signal transduction pathways by a family of proteins comprising five structurally related members PIAS1/GBP, PIAS3, PIASxα, PIASxβ and PIASγ. They were discovered using a two hybrid screen for STAT interacting proteins, and subsequently termed the *p*rotein *i*nhibitor of *a*ctivated *S*TAT (PIAS) family (Chung *et al.*, 1997; Liu *et al.*, 1998). Each of the PIAS proteins contain a central zinc finger and an acidic transactivation domain at the C-terminal (Chung *et al.*, 1997; Liu *et al.*, 1998; Sturm *et al.*, 2000). These proteins prevent DNA recognition by binding to phosphorylated STAT dimers, thus inhibiting STAT mediated signaling cascades (Shuai, 2000). PIAS1 was

discovered as a specific binding partner of activated STAT1, but not of STAT2 or STAT3 *in vivo* (Liu *et al.*, 1998). This interaction between PIAS1 and STAT1 was dependent on Tyr^{701} phosphorylation of STAT1, as the interaction was only observed in samples after IFN treatment, which in turn phosphorylated STAT1 (Liu *et al.*, 1998). It was also demonstrated that PIAS1 was able to inhibit the activity of STAT1 through a series of luciferase reporter assays where the activation of STAT1 induced genes was significantly decreased (Liu *et al.*, 1998). PIAS3 was cloned by its similarity to PIAS1 (Chung *et al.*, 1997) and has since been demonstrated to interact with phosphorylated STAT3 and not STAT1 (Liu *et al.*, 1998). The interaction between the PIAS proteins and STAT molecules is dependent on cytokine stimulation, as their affinity is much greater for tyrosine phosphorylated STATs. The actual interaction is unknown, however due to the lack of phosphotyrosine domains in the PIAS proteins, a conformational change in the phosphorylated STATs may result in the exposure of PIAS interacting domains (Liu *et al.*, 1998). Blocking STAT activity either by preventing STAT dimerisation or blocking the STAT proteins from binding to DNA in promoter regions of STAT dependent genes are two possible mechanisms of inhibition.

1.8.3 Suppressors of Cytokine Signaling (SOCS)

Socs1 was discovered by three independent groups hence the three different nomenclatures: SOCS1, JAB and SSI1. SOCS1 was discovered as an inhibitor of IL6 function by an expression cloning approach, utilising the murine monocytic leukaemic M1 cell line. M1 cells that had been retrovirally infected with a cDNA expression library, and were unresponsive to the antiproliferative effects of IL6 were selected in a semi solid agar culture by their inability to generate compact colonies with the addition of IL6. From these IL6 unresponsive clones a 1.4 kb cDNA insert was recovered by PCR from the retrovirus that had integrated into the genomic DNA of the clones (Starr *et al.*, 1997). JAB was identified using a yeast two hybrid system looking for proteins that bound to the tyrosine kinase domain (JH1) of JAK2 (Endo *et al.*, 1997). SSI1 was identified by generating an antibody which recognised a common sequence of the SH2 domain of STAT3 (GTFLLRFS). This antibody was used

to screen a murine thymic cDNA library from which an unknown gene containing a SH2 domain was isolated and later termed SSI1 (*S*TAT induced *S*TAT *i*nhibitor) (Naka *et al.*, 1997). Subsequently, database searches have revealed a family of SOCS proteins. Of these proteins, CIS, a previously identified *c*ytokine-*i*nducible *S*H2 containing protein, was highly homologous to SOCS1. In addition to CIS, six other family members, SOCS2-7 were also found to be closely related to SOCS1 and hence comprise the SOCS protein family.

Each SOCS family member contains a conserved SH2 domain and a 40 amino acid C-terminal region of homology termed the "SOCS box". Utilising the C-terminal SOCS box in a database search, further SOCS box containing proteins were revealed. These proteins did however contain different recognisable motifs in place of the SH2 domains found in the SOCS proteins and have been named accordingly. Proteins containing WD-40 repeats were termed WSB (WSB1 and 2), proteins containing ankyrin repeats were termed ASB (ASB1-ASB3), those containing SPRY domains were termed SSB (SSB1-SSB3). Figure 1.13 illustrates the different SOCS box containing proteins with their different N-terminal domains.

1.8.4 SOCS Induction by Cytokines

Studies have shown that *Socs1* is induced by growth *h*ormone (GH) (Ram & Waxman, 1999; Adams *et al.*, 1998; Favre *et al.*, 1999), type I and II IFNs (Naka *et al.*, 1997; Sakamoto *et al.*, 1998; Song & Shuai, 1998), IL4 (Endo *et al.*, 1997; Losman *et al.*, 1999; Haque *et al.*, 2000), IL6 (Endo *et al.*, 1997; Starr *et al.*, 1997; Naka *et al.*, 1997), *th*rombo*po*ietin (TPO) (Naka *et al.*, 1997) and *pro*lactin (PRL) (Helman *et al.*, 1998; Pezet *et al.*, 1999) and can also then in a negative feedback mechanism, inhibit their signaling. Similarly *Socs3* is induced by and subsequently able to inhibit signaling by GH (Ram & Waxman, 1999; Adams *et al.*, 1998; Favre *et al.*, 1999; Davey *et al.*, 1999), *e*rythro*po*ietin (EPO) (Naka *et al.*, 1997; Masuhara *et al.*, 1997), GM-CSF (Masuhara *et al.*, 1997), type I and II IFNs (Sakamoto *et al.*, 1998; Song & Shuai, 1998), IL6 (Starr *et al.*, 1997; Nicholson *et al.*, 1999), leptin (Bjorbaek *et al.*, 1998), *l*eukemia *i*nhibitory *f*actor

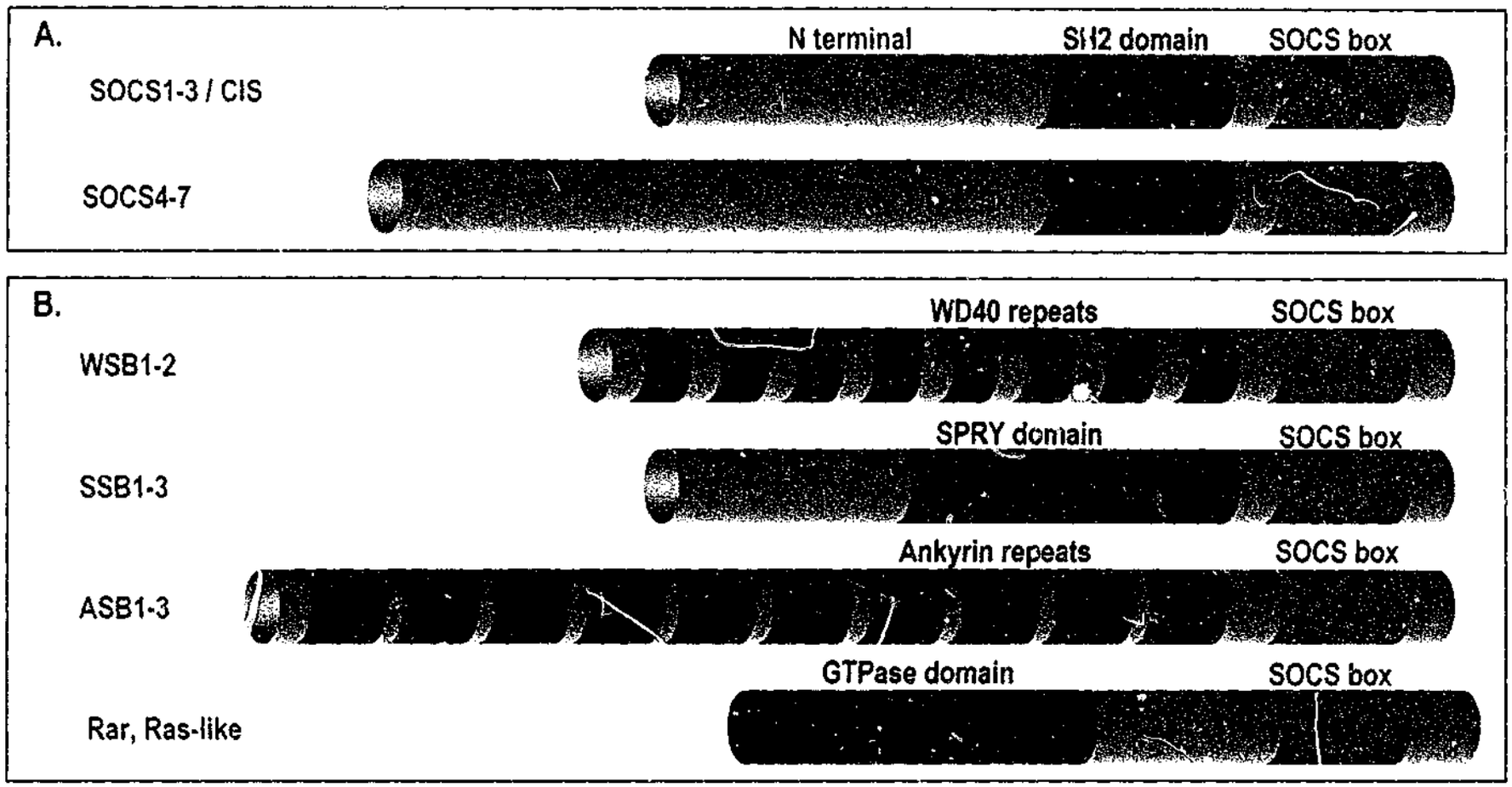


Figure 1.13: SOCS Protein Family Structure.

A. Each of the SOCS protein members SOCS1-7 and CIS all posses an SH2 domain and a C-terminal SOCS box domain.

B. Related SOCS family members all possess a C-terminal SOCS box motif, but no SH2 domain, in place of this are other domains including WD40 repeats, ankyrin repeats a SPRY domain or a GTPase domain.

(LIF) (Masuhara *et al.*, 1997; Minamoto *et al.*, 1997; Bousquet *et al.*, 1999)and PRL. (Helman *et al.*, 1998; Pezet *et al.*, 1999). In contrast both SOCS2 and CIS are induced by growth hormone (Karlsson *et al.*, 1999; Horvat & Medrano, 2000), with LIF, OSM and CNTF also inducing the expression of *Socs2* (Turnley *et al.*, 2002).

The mechanism of induction of the *Socs* genes is predominantly via JAK/STAT signaling. However, it is becoming evident that *Socs* expression can also be induced by factors that do not signal through STATs, such as LPS (Stoiber *et al.*, 1999), or through JAKs including insulin and stem cell factor (SCF) (De Sepulveda *et al.*, 1999; Emanuelli *et al.*, 2000). LPS and CpG DNA acting through TLR4 and TLR9 in macrophages respectively induce both *Socs1* and *3* (Stoiber *et al.*, 1999; Crespo *et al.*, 2000; Dalpke *et al.*, 2001; Kinjyo *et al.*, 2002). Then, similarly to other factors that induce *Socs1* and *3*, the proteins are capable of acting in a negative feedback loop to switch off LPS signaling through both direct and indirect mechanisms (Nakagawa *et al.*, 2002). These studies thus demonstrate that SOCS1 is a key regulatory component of the innate immune system (Kinjyo *et al.*, 2002).

Basal expression levels of the SOCS proteins differ between family members. In mice, CIS is highly expressed in kidney, muscle, liver, fat, hypothalamus and heart. In contrast SOCS1 is expressed mainly in the thymus and to a lesser degree the spleen (Starr *et al.*, 1997; Minamoto *et al.*, 1997; Tollet-Egnell *et al.*, 1999). SOCS2 is detected in heart, lungs, spleen and liver while SOCS3 is detected in lungs, spleen and fat (Starr *et al.*, 1997; Minamoto *et al.*, 1997; Tollet-Egnell *et al.*, 1999). In response to the majority of cytokine stimuli, SOCS1 and 3 appear to have similar inducible expression patterns being induced quite dramatically, while SOCS2 and CIS appear to have less dramatic inducible expression profiles in response to cytokine stimulation.

The *Socs1* promoter contains binding sites for STAT1, 3 and 6, consistent with *Socs1* inducibility by STAT activation (Schluter *et al.*, 2000; Naka *et al.*, 1997; Saito *et al.*, 2000). However, the mouse *Socs1* 5' untranslated region (UTR) contains two AUGs (translation initiation sites) upstream of the *bona fide* AUG

which can regulate *Socs1* gene expression through translational repression (Schluter *et al.*, 2000; Gregorieff *et al.*, 2000). 5' UTRs are able to down regulate gene translation by blocking ribosomal binding and movement with the formation of stable secondary structures (Gregorieff *et al.*, 2000). Therefore *Socs1* appears to be regulated not only by the levels of activated transcription factors (e.g.. STATs) but also by translational mechanisms.

1.8.5 SOCS Proteins Inhibit Cytokine Signaling Pathways

It is clear that when CIS, SOCS1 and 3 are overexpressed, they inhibit signaling via the JAK/STAT pathway. SOCS1 has been shown to directly interact with JAK1, JAK2 and TYK2 and inhibit in phosphorylation. This interaction results in a loss of activity and the subsequent down-regulation of JAK dependent phosphorylation of both receptors and STAT molecules (Endo *et al.*, 1997; Naka *et al.*, 1997; Nicholson *et al.*, 1999). SOCS1 binds via its SH2 domain to the activation loop of the JAKs. However, in order to inhibit the phosphorylation of the JAKs, a region immediately N-terminal of the SH2 domain of approximately 24 residues is required (Nicholson *et al.*, 1999; Yasukawa *et al.*, 1999). In contrast to SOCS1, CIS is believed to compete with STAT proteins for the phosphorylated receptor residues, again inhibiting signaling at the receptor level. Through a series of mutation studies of the phosphotyrosine docking sites of the EPO receptor, it appears that CIS may be functioning by a mechanism other than competition for docking sites on activated receptors. SOCS3 binds to JAK2 albeit to a lesser degree than SOCS1, but does not inhibit the *in vitro* kinase activity of JAK1 or JAK2 when overexpressed, suggesting that it elicits its effects by a different mechanism to SOCS1. However, similar to CIS, SOCS3 is able to bind to phosphorylated receptors, among these being GH receptor, gp130, the IL12β subunit, leptin and the EPO receptors. Studies with the GH receptor and also with the IL12β receptor subunit have linked the *in vitro* data to the *in vivo* data suggesting that SOCS3 will inhibit the kinase activity of JAK2 when bound to an activated receptor

(Sasaki *et al.*, 1999). Although there are similarities between CIS, SOCS1 and SOCS3 they appear to inhibit signaling pathways via different mechanisms.

The mechanisms involved in how the SOCS proteins regulate signaling pathways have begun to be elucidated. The SH2 domains of these proteins, along with the SOCS box that defines the entire family, have different but important roles. The SOCS SH2 domains bind to the SH2 domains of activated receptors/JAKs. A series of experiments illustrated the importance of the SOCS box by generating a mouse that had the SOCS box from the *Socs1* gene deleted. These experiments demonstrated that only partial inhibition was possible by the binding of the SH2 domains (Narazaki *et al.*, 1998; Nicholson *et al.*, 1999; Yasukawa *et al.*, 1999). Full inhibition requires a functional SOCS box to act as a target for proteosomal degradation (Zhang *et al.*, 2001). This occurs as the SOCS box extends into the cytoplasm that binds to elongin C that, in turn binds elongin B, and through the elongin B ubiquitin-like domain, subsequently binds to the proteosome. These associations target the SOCS protein along with bound proteins (JAKs and/or cytokine receptors) for proteosomal degradation, leaving the cell ready to respond to stimuli once more (Kaelin & Maher, 1998; Conaway *et al.*, 1998) (Figure 1.14).

1.8.6 SOCS Knockout Models

The generation of mouse knockout models for each of the SOCS proteins has demonstrated specific and non-redundant functions for each family member. Table 1.3 outlines the main phenotypes observed of the SH2 domain containing family members. Of interest to this study is the phenotype of the *Socs1*$^{-/-}$ mouse. These mice exhibit stunted growth and die during the neonatal period from a disease characterised by fatty degeneration of the liver, severe lymphopenia and cellular infiltrates of pancreas, lungs and heart (Starr *et al.*, 1998; Naka *et al.*, 1998). This phenotype was demonstrated to be a result of a hypersensitivity to IFNγ as the generation of *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice ablated the neonatal lethality (Alexander *et al.*, 1999). Marine *et al.* (1999) demonstrated that there was an increase in IFNγ production resulting in excessive activation of

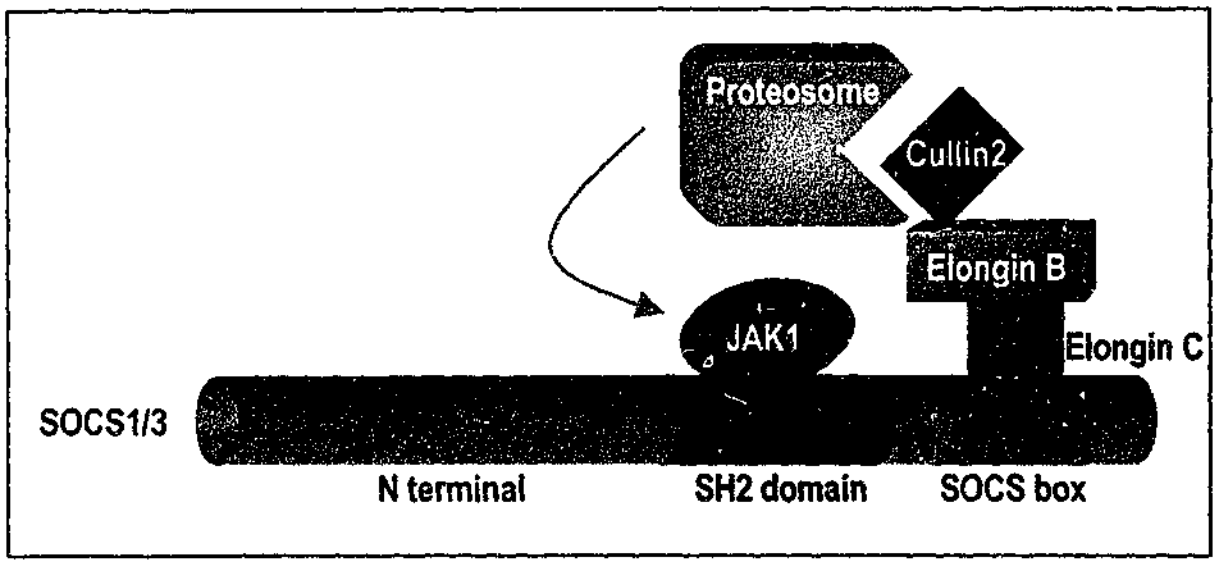


Figure 1.14: Model of SOCS box function.

SOCS1/3 binds phosphorylated tyrosines on JAKs via their SH2 domains and subsequently inhibit further JAK kinase activity. Elongin C binds to the SOCS box and recruits elongin B and cullin2 which target bound proteins to proteosomal degradation.

Table 1.3: *Socs*$^{-/-}$ Mice Phenotypes.

(Taken from Levy & Darnell, 2002)

SOCS Protein	Phenotype
SOCS1	Perinatal lethality owing to unopposed IFNγ induced liver degeneration.
SOCS2	Gigantism owing to unopposed signaling by growth hormone and IGF1.
SOCS3	Embryonic lethality owing to multiple placental and haematopoietic defects.
CIS	No phenotype of null, however enhanced T cell signaling in mice overexpressing CIS.

T cells, which appeared to be the main infiltrating cell type in the pathology of the *Socs1*$^{-/-}$ mice.

Mice that are deficient for *Socs2* are larger in size compared to their wild-type littermates due to a hyper-responsiveness to GH and *i*nsulin-like *g*rowth *f*actor 1 (IGF1) (Metcalf *et al.*, 2000). *Socs3*$^{-/-}$ mice are fetal lethal due to a defect in fetal erythropoiesis, indicating that SOCS3 plays an important regulatory role in erythropoietin signaling pathways (Marine *et al.*, 1999).

The fact that so many cytokines are able to induce SOCS1 makes it difficult to decipher a specific biological role for this protein. The phenotype of the *Socs1*$^{-/-}$ mouse has demonstrated that SOCS1 has an irreplaceable role in IFN signaling. Most data thus far has been directed towards understanding the link between SOCS1 function and IFNγ. However in light of the interactions between type I and II IFN signaling, biological effects and the cross talk that occurs, it is likely that SOCS1 plays a role in type I IFN signaling. The phenotype of the *Socs1*$^{-/-}$ mice emphasises the potential hazards of excessive IFNγ signaling and serves as an important reminder of the need to balance or limit this signal. To date there is little published work addressing the roles of SOCS1 in the regulation of type I IFN responses, particularly *in vivo*, which will be the focus of this study.

This literature review has addressed the IFN family, their cognate receptors and how they function. Therefore by fully understanding how the IFN system functions, the more likely it will become that the system can be manipulated in such a way that it will alleviate the unfavourable side effects of IFN treatment mentioned earlier

1.9 RATIONALE AND OBJECTIVES OF THIS PROJECT

The pleiotrophic actions of type I IFNs led to early recognition of their therapeutic potential which clinical trials have shown promising results in the treatment of viral infection, cancers and multiple sclerosis. However the adverse side effects that accompany most current IFN treatments highlight the importance of balancing the positive with the negative effects to achieve beneficial IFN signals. Therefore by utilising genetically modified mice of IFN associated factors, the importance of the IFN system in disease including infection, cancer and inflammation can be studied *in vivo*. An improved understanding of the factors which control IFN responses, will be important to improving the therapeutic actions of these widely used drugs and to the improvements in other treatments which may be affected by IFNs.

At the commencement of this study the mechanisms of IFN signaling were known, however little was known on how IFN signaling was regulated. With the discovery of the SOCS family of cytokine proteins and their ability to inhibit numerous cytokine signaling pathways, the role of these proteins, in particular SOCS1 in type I IFN signaling became the focus of this study as previously nothing was known. These proteins may have an effect on how IFN signaling is switched off, therefore to investigate this, overexpressing SOCS1, 2, 3 and CIS cell lines were utilised for *in vitro* studies to examine the effect of these proteins on IFN signaling. In order to study the *in vivo* effects the generation of the *Socs1*$^{-/-}$ mice was utilised along with the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice, which were rescued from the neonatal lethality of the *Socs1*$^{-/-}$ mutation.

Analysis of type I IFN signaling could then be extended with the use of both the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice which, present with different phenotypes however thus far there is a lack in the knowledge of different signaling pathways initiated by the different receptor subunits. With the use of *Socs1*$^{-/-}$ mice together with the type I IFN receptor knockout mice, signaling pathways that could not be observed could be highlighted with the lack of a negative regulator. Therefore this body of work could elucidate mechanisms of how type I IFN signaling is

regulated which could improve existing IFN therapies to generate more beneficial treatments than those that currently exist.

The main aims of this project were:

- To examine what role the SOCS1 protein plays in the regulation of the type I IFN responses.

- To elucidate molecular mechanisms that SOCS1 utilises to regulate the signaling of the type I IFNs with the use of various animal models.

- To elucidate the differences in the signaling capabilities between the *Ifnar1*$^{-/-}$ and the *Ifnar2*$^{-/-}$ mice with the aim of uncovering alternate signaling pathways that may be specific to each chain of the receptor.

CHAPTER 2

Materials and Methods

2.1 LIST OF SOLUTIONS, SUPPLIERS, EQUIPMENT, OLIGONUCLEOTIDES AND PLASMIDS

Appendix A Buffers and Solutions

Appendix B List of Suppliers

Appendix C List of Equipment

Appendix D Oligonucleotides

Appendix E Plasmid Sources

2.2 DNA MANIPULATIONS

2.2.1 Generation of Templates for use as Probes

To generate probes from plasmids containing cDNA fragments of known interferon regulated genes for Northern and Southern hybridisations, the DNA was transformed directly into bacteria for the production of large amounts of DNA. Following this, the fragments were purified following restriction enzyme digestion as outlined below. To generate templates for Real Time PCR, small fragments (100-260 bp) were amplified out of each of the plasmids containing the genes of interest by PCR.

2.2.1.1 Template Amplification by Polymerase Chain Reaction (PCR)

PCR was performed to amplify 5 DNA fragments required for use as templates in Real Time PCR protocols. PCR reactions were carried out in 50 µl volumes consisting of 5 µl 10 x PCR thermophilic DNA polymerase buffer (*Promega*), 4 µl $MgCl_2$ (25mM) (*Promega*), 1 µl dNTPs (10 mM) (*Promega*), 0.25 µl *Taq* DNA Polymerase (5 U/µl) (*Promega*), 36.75µl dH_2O, 1 µl cDNA (50 ng), then 1 µl of each sense and antisense primer (200 ng/µl) (*Proligo*) for each corresponding gene (refer to Appendix D). The PCR was performed using the GeneAmp PCR system 2400 (*Applied Biosystems*) with 1 cycle of 5 minutes at 94°C, 35 cycles

of denaturing (1 minute at 94°C), annealing (1 minute at 58°C), extension (1 minute at 72°C) followed by 1 cycle at 72°C for 7 minutes. One negative control was included which contained no DNA. The fragments were subsequently electrophoresed through a 1.5% agarose gel and purified by an agarose gel purification kit (*Qiagen*) (refer to 2.2.1.6 and 2.2.1.7).

2.2.1.2 Ligation

The fragments amplified by PCR were cloned into pGEM®-T vectors (*Promega*) in order to generate larger amounts of plasmid DNA for use as templates in Real Time PCR. The ligation reactions were completed in 30 µl volumes consisting of 1 µl pGEM®-T vector (50 ng/µl) (*Promega*), 1-4 µl of insert (4-20 ng total), 15 µl of 2x rapid ligation buffer (*Promega*), 2 µl T4 DNA ligase (3 U/µl) (*Promega*) and 8-11 µl dH_2O. The reaction mixes along with appropriate controls were incubated overnight at room temperature. The following day the ligation mixes were transformed into JM109 bacteria (*Promega*) (refer to 2.2.1.3).

2.2.1.3 Transformations using JM109 Cells

Commercial competent JM109 bacteria (*Promega*) were transformed by heat shock with 50 ng plasmid DNA. Bacteria were heat treated for 45 seconds at 42°C then immediately cooled on ice for 2 minutes. SOC media (380 µl) (Appendix A) was added and the bacteria were placed in a 37°C shaking incubator for 30 minutes to recover. These were then plated out onto LB agar plates containing 50 mg/ml ampicillin (*CSL*) and grown at 37°C overnight.

2.2.1.4 Mini/Maxi DNA Preparations

Single colonies resulting from the transformations grown overnight at 37°C were picked and placed in 1 ml of LB broth (Appendix A) containing 50 mg/ml ampicillin (*CSL*) and again grown overnight at 37°C in a shaking incubator. Plasmid DNA was isolated using the alkaline lysis method (Sambrook *et al.*,

1987) and restriction enzyme digests performed to confirm that they contained the desired insert. Once the insert was confirmed, maxi preparations of plasmid DNA were prepared from 250 ml TB (Appendix A) cultures and following the alkaline lysis method, plasmid DNA was purified using a cesium chloride gradient (Sambrook *et al.*, 1987). The absorbances at 260 nm and 280 nm were measured to calculate the concentration of DNA using OD_{260} x 50 (DNA constant) x 100 (dilution factor) = ng/µl of DNA recovered. The $OD_{260/280}$ ratio was used as a measure of the purity of the DNA recovered with a ratio of 1.6-1.8 considered pure.

2.2.1.5 Restriction Enzyme Digests

DNA digestions were conducted as appropriate for the specific construct and analysed by agarose gel electrophoresis (see 2.2.1.6). Digests were performed according to the manufacturer's instructions, generally using 1 x restriction enzyme buffer and 1 unit enzyme per µg of DNA. Initially a diagnostic digest was performed where 400 ng of DNA plasmid was digested to confirm that the correct insert was present. Subsequently, 20 µg preparative digests with 20 units of enzyme were conducted to obtain sufficient quantities of probe templates. Promega supplied all enzymes used in this project unless otherwise stated

2.2.1.6 Agarose Gel Electrophoresis

Horizontal mini or wide mini Sub™ cell agarose gel apparatus (*BioRad*) were used for electrophoresis. Agarose gels were prepared using agarose (*Promega*), 1 x TAE buffer (Appendix A) and 25 µg ethidium bromide (EtBr). Generally, 1% agarose gels were utilised for DNA fragments greater than 500 bp in size, while for fragment sizes less than 500 bp, 2% agarose gels were used. DNA samples containing 10% of gel-loading dye (Appendix A) were loaded into the wells in the gel and electrophoresed at 70-100 volts in 1 x TAE buffer (Appendix A). The DNA was visualised under ultra-violet (UV) light and photographed using the BioRad Gel Doc 1000 (*BioRad*).

2.2.1.7 Recovery of DNA Fragments from Agarose Gels

DNA fragments generated by restriction enzyme digestions of plasmid DNA were separated by agarose gel electrophoresis. The appropriate DNA band was excised from the gel using a scalpel blade under UV illumination. The DNA fragment was purified using a QIAEX II™ Agarose Gel Extraction kit according to the manufacturer's specifications (*Qiagen*). The concentration of DNA was estimated following electrophoresis on an agarose gel by comparison with the DNA standards of known concentration.

2.2.2 DNA Labelling by Random Priming

A random primed DNA labeling kit (*Roche*) was used to label double stranded DNA (dsDNA) fragments with α-^{32}P dCTP (1 mCi/ml) (*Amersham Pharmacia*). The labeling reaction involved placing 25 ng of probe template into 9 µl 1 x TE (Appendix A) and denatured by boiling for 5 minutes after which the reactions were briefly spun then incubated on ice for a further 2 minutes. Following this 2 µl of hexanucleotide mix (*Roche*), 3 µl dNTPs-C (without dCTP) (10 mM) (*Promega*) and 1 µl Klenow enzyme (5 U/µl) (*Roche*) were added. To the reaction mixes, 5 µl of α-^{32}P dCTP was added and the reactions incubated for a further 30-40 minutes at 37°C. Following these reactions, the ^{32}P-labeled fragments (probes) were purified by chromatography through a Sephadex™ G-50 NICK column (*Amersham Pharmacia*). The specific activities of the probes were measured in a 1900TR Liquid Scintillation β counter (*Canberra Packard*) then 1 x 10^6 cpm/ml for Southern hybridisation and 2 x 10^6 cpm/ml for Northern hybridisation were added to 200 µl salmon sperm DNA (ssDNA) (*Roche*). The probes plus the ssDNA were added to the hybridisation solutions (Appendix A). Once the purified ^{32}P-labeled fragments had been added to the membranes they were left to incubate at 65°C for Southern and 42°C for Northern hybridisations overnight.

2.2.3 Genotyping

Throughout this project various mouse models were utilised which needed to be genotyped. The three genes which needed to be checked whilst maintaining mouse colonies were *Ifnar1*, *Ifnar2* and *Socs1*.

2.2.3.1 Isolation of Genomic DNA

Half a centimetre of mouse tail was digested overnight at 55°C with 600 µl of denaturing tail lysis buffer (Appendix A) and 15 µl of Proteinase K (14 mg/ml) (*Roche*). An equal volume of phenol:chloroform was added, mixed, then centrifuged in a Sigma benchtop centrifuge for 8 minutes at 13000 rpm. The upper aqueous phase was harvested and re-extracted with phenol:chloroform as above, then extracted further with an equal volume of chloroform. The mixture was incubated with 1 µl *RNaseA* (10 mg/ml) (*Roche*) at 37°C for 1 hour to digest RNA. Genomic DNA was then precipitated with 1 ml 100% ethanol (EtOH), spooled with a sealed glass Pasteur pipette, washed in 70% EtOH and resuspended in 150 µl 1 x TE buffer (Appendix A). The amount of DNA was subsequently estimated against a known amount of lambda *Hin*dIII by agarose gel electrophoresis (refer to 2.2.1.6)

2.2.3.2 Southern Hybridisation Analysis

All genotyping for the *Ifnar1* and *Socs1* gene status of the mice used throughout this project were determined by Southern hybridisation analysis. DNA extracted from the tails of the mice (refer to 2.2.3.1) were digested, 20 µg with 20 units of *Eco*RI enzyme (*Promega*) and 7 µl of 10x multicore buffer (this volume of buffer has been demonstrated to give optimal conditions for a reaction of a total volume of 50 µl) (*Promega*) (refer to 2.2.1.5). Digests incubated overnight at 37°C were subsequently electrophoresed on 1% agarose (*Promega*) gels (refer to 2.2.1.6) and transferred onto GeneScreen Plus membranes (*NEN Life Science Products*) using capillary action with 0.4 M NaOH buffer (Appendix A). Following transfer the membranes were rinsed in 2 x SSC (Appendix A) then

dried at 55°C for 40 minutes. Membranes were placed into hybridisation bottles with pre-hybridisation solution (Appendix A) for 2 hours at 65°C.

The hybridisation probes were prepared as described in section 2.2.1, then radioactively labeled as outlined in section 2.2.2. Following hybridisation, the membranes were washed with a series of washes with increasing stringency. Two washes of 2x SSC/0.1% SDS for 15 minutes each at 65°C were followed by a further 2 washes of 0.1x SSC/0.1% SDS for 15 minutes each at 65°C. The membranes were removed from the hybridisation bottles and enclosed in plastic to be exposed to Phosphorimage screens overnight at room temperature. Following exposure, the Fuji phosphorimage screens were developed in the FLA-2000 phosphorimager (*Fujifilm*) using Image Gauge software.

2.2.3.3 Genotyping by Polymerase Chain Reaction (PCR)

PCR was used to determine the genotype of *Ifnar2* allele. The PCR reactions were carried out in a 50 µl volume consisting of approximately 100 ng of genomic DNA, 5 µl Buffer 1 (*Stratagene*), 2.5 µl Dimethyl Sulphoxide (DMSO) (*BDH*), 1.25 µl dNTPs (10 mM) (*Promega*), 1 µl of each primer: Ifnar 2f and Ifnar 2r (200 ng/µl) (Appendix D) (*Proligo*), 0.5 µl *Taq* DNA Polymerase (5 U/µl) (*Promega*) and then made up to volume with dH_2O. The PCR mix was then subjected to 1 cycle of pre-denaturation (94°C, 5 minutes), followed by 30 cycles of denaturation (94°C, 1 minute), annealing (58°C, 1 minute), extension (72°C, 3 minutes) and then 1 final cycle at 72°C for a further 5 minutes on GeneAmp PCR system 2400 (*Applied Biosystems*). Three positive controls of known genotype were included as well as one negative control containing no DNA. PCR products were subsequently visualised on a 1% agarose gel (see 2.2.1.6).

2.3 RNA MANIPULATIONS

All solutions used to extract poly (A)+ RNA and total RNA were treated with DEPC (*Sigma*) prior to use in order to inhibit RNase activity. All solutions and buffers used throughout are listed in Appendix A.

2.3.1 Poly (A)+ RNA Extraction

Snap frozen cells/organs were homogenised using an Ultra-Turrax T25 homogeniser (*Crown Scientific*) following the addition of 300 µl Proteinase K (14 mg/ml) (*Roche*) and 25 ml extraction buffer for 2-5 minutes. Following a 30 minute incubation in a 55°C H_2O bath, samples were removed and allowed to cool to room temperature. Once at room temperature, 2 ml 5 M NaCl plus 2.5 ml of Oligo dT Cellulose (*Roche*) in binding buffer were added and the samples were gently rotated at room temperature for 1-3 hours, then centrifuged at 1000 rpm for 5 minutes. The Oligo dT Cellulose pellet was washed twice with 10 ml binding buffer and once with 10 ml wash buffer. Elution of the poly (A)+ RNA was achieved by addition of 2 ml of elution buffer to the Oligo dT Cellulose, followed by gentle mixing and incubation in a 60°C H_2O bath for 5 minutes. The Oligo dT Cellulose was pelleted at 1000 rpm for 2 minutes, the supernatants removed and transferred to a fresh tube containing phenol:chloroform (1:1). A further 1.5 ml of elution buffer was added to the Oligo dT Cellulose pellets and incubated again for 5 minutes at 60°C. The Oligo dT Cellulose again was pelleted at 1000 rpm for 2 minutes and the supernatant was added to the tubes containing the phenol:chloroform (1:1) with the 2 ml of supernatant from the previous elution. This mixture was gently shaken and centrifuged (*Heraeus Instruments*) at 5000 rpm for 5 minutes. The aqueous phase was transferred to 4 ml of chloroform, gently shaken and centifuged (*Heraeus Instruments*) at 5000 rpm for 5 minutes. The aqueous phase was then added to 8 ml 100% EtOH and 600 µl 3 M sodium acetate (NaOAc) pH 5 followed by overnight incubation at -20°C to allow the poly (A)+ RNA to precipitate. Following centrifugation (*Beckman Instruments*) at 10,000 rpm for 1 hour the supernatant was removed and the RNA pellet was then resuspended in 200 µl RNA grade 1 x TE. Once resuspended, 10 µl was removed for quantitation by spectrophotometry in 400 µl RNA grade 1 x TE while the remaining 190 µl was added to 550 µl 100% EtOH plus 19 µl 3 M NaOAc and stored at -80°C.

The quantitation of the mRNA was achieved by spectrophotometry (*Perkin Elmer*) at a wavelength of 260nm, where the following equation was used to obtain total mRNA recovered:

OD_{260} x 304 (equation constant) = total µg of mRNA.

The equation constant takes into account the dilution factor and the final volume of the mRNA sample.

2.3.2 RNA Gel Electrophoresis

RNA samples were electrophoresed on gels containing 1% agarose, 0.63% formaldehyde (v/v), 1 x MOPS and dH_2O. In order to visualise the RNA, 2.5 µl of 10 mg/ml EtBr (*BioRad*) was added to the gel mix. The gels were electrophoresed at 80 volts in 1 x MOPS. All gel tanks, gel trays and combs used were first treated with DEPC H_2O in order to remove any RNases.

Aliquots of the poly (A)+ RNA containing 3 µg of mRNA each were mixed together with 20 µg of transfer-RNA (tRNA) (*Promega*), acting as a carrier, and then centrifuged at 13,000 rpm for 15 minutes in a microcentrifuge (*Sigma*). The RNA pellet was dried and subsequently resuspended in 10 µl RNA-suspension buffer (RSB) and placed at 65°C for 5 minutes after which 2 µl RNA-loading dye was added. The samples were then loaded into the wells of the gel.

2.3.3 Northern Hybridisation Analysis

RNA was transferred overnight by capillary action from the agarose-formaldehyde gel onto a Hybond-C Extra nitrocellulose membrane (*Amersham Life Science*) with 20x SSC. Prior to transfer the Hybond-C was pre-wet with dH_2O then soaked in the transfer buffer for at least 15 minutes. Following overnight transfer, the membrane was baked for 2 hours at 80°C in a vacuum oven. The membrane filter was pre-hybridised for 2-3 hours at 42°C in 50% formamide, 5x SSC, 1x Denhardt's solution and 10 mg/ml ssDNA (denatured by boiling for 5 minutes) (*Roche*). Filters were then hybridised with a ^{32}P-labeled

probe (section 2.2.2), in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulphate and 50 µg/ml of ssDNA (sheared) for at least 16 hours at 42°C. Filters were sequentially given 15 minute washes once in 2x SSC/0.1% SDS at 65°C, twice in 0.2x SSC/0.1% SDS at 65°C and twice in 0.1x SSC/0.1% SDS at 65°C. Filters were first exposed to Kodak X-ray film with intensifying screens at -80°C. For quantitation the filters were then exposed to a Fuji Phosphorimage screen at room temperature overnight, developed using a FLA-2000 phosphorimager (*Fujifilm*) and analysed using Image Gauge software.

2.3.3.1 Membrane Stripping

A solution of boiling 0.1% (v/v) SDS was added to the membrane and allowed to cool to room temperature. The membrane was then exposed to a Fuji Phosphorimage screen for 2-3 hours, developed using a FLA-2000 Phosphorimager (*Fujifilm*) and analysed using Image Gauge software to confirm the removal of the radioactive probe.

2.3.4 Microarray Analysis

RNA samples for microarray analysis during this project were extracted from bone marrow derived macrophages (BMMs) (refer to section 2.5.2) which were cultured from *Ifnar1*$^{-/-}$, *Ifnar2*$^{-/-}$ and wild-type mice.

2.2.5.1 Total RNA Extraction

RNA was extracted using a RNeasy Midi Kit for isolation of total RNA from animal cells (*Qiagen*). All RNA samples were thawed to room temperature after being stored at -20°C after harvesting (refer to section 2.5.5). All subsequent procedures were carried out at room temperature (20°C). Once thawed, 1 volume (4 ml) of 70% EtOH was added to each lysate and mixed thoroughly by shaking vigorously. Half of the sample was added to a RNeasy midi column and centrifuged (*Heraeus Instruments*) for 5 minutes at 5000 g. The supernatant was discarded and the remainder of the sample added to the

column that was spun again after which the supernatant was discarded again. Subsequently 4 ml of Buffer RW1 (*Qiagen*) was added to the RNeasy column (*Qiagen*) and centrifuged (*Heraeus Instruments*) at 5000 g for 5 minutes. After discarding the supernatant once again, 3 ml of Buffer RPE (*Qiagen*) was added to the RNeasy column and centrifuged (*Heraeus Instruments*) for 5 minutes at 5000 g. This wash step was repeated again with 2 ml of Buffer RPE (*Qiagen*). The RNeasy columns were then transferred to fresh tubes. To elute the RNA, 250 µl of RNase-free H_2O was added to each column and incubated at room temperature for 1 minute. Columns were then centrifuged (*Heraeus Instruments*) at 5000 g for 3 minutes. The elution step was repeated twice to obtain RNA in a total volume of 750 µl.

To quantitate the RNA and to test the quality, 10 µl was diluted into 60 µl of H_2O the OD_{260} measured on a spectrophotometer (*Perkin Elmer*). A further 10 µl was electrophoresed on a 1% native agarose (*Promega*) gel. The amount of RNA was calculated from the OD reading at 260nm with the equation:

OD_{260} x 40 (RNA constant) x dilution factor = µg/ml of RNA recovered.

Ethanol and NaOAc were added to 30-50 µg aliquots of RNA for subsequent microarray analysis. These samples were stored at -20°C. The remaining RNA also had EtOH and NaOAc added but were stored at -80°C.

2.3.4.2 Indirect Labeling Procedure

Preparation of cDNA

The RNA was centrifuged (*Sigma*) for 30 minutes at 13000 rpm at 4°C after which the supernatant was discarded and the pellets washed in 70% EtOH. Pellets were then centrifuged again to remove any residual EtOH after which pellets were left to air dry.

The dried RNA pellet was resuspended in 9.5 µl RNase free H_2O. To this, 2 µl of Oligo dT primer (2 µg/µl) (*Proligo*) was added and incubated for 10 minutes at 70°C. Samples were incubated on ice for 5 minutes. To each sample 6 µl of 5x first strand buffer (*Invitrogen*), 3 µl of 0.1% DTT (*Invitrogen*), 1.5 µl of a dGTP/dATP/dCTP mix (10 mM each) (*Invitrogen*), 3.6 µl of 2.5 mM dTTP

(*Invitrogen*), 2.4 µl of 2.5 mM amino-allyl UTP (*Sigma*) and 2 µl (200 U/µl) of Superscript II Reverse Transcriptase enzyme (*Invitrogen*) was added. The samples were then incubated at 42°C for 1 hour.

Purification of cDNA

The RNA was hydrolysed by the addition of 1.5 µl of 20 mM EDTA (filter sterilised) and 1.5 µl of 1 N NaOH. Samples were incubated at 70°C for 10 minutes. The reaction was then buffered with the addition of 1.5 µl of 1 N HCl. In a fresh eppendorf tube 450 µl of Piero's binding buffer was combined with 45 µl of stock solution. The cDNA was then added to the binding buffer/stock solution mix and subsequently loaded onto the columns from a GFX PCR DNA and Band Purification Kit (*Amersham Pharmacia*). Columns were centrifuged (*Sigma*) at room temperature at 15,000 rpm for 3 minutes after which the supernatant was discarded. The columns were then washed with 500 µl of wash buffer (*Amersham Pharmacia*) and centrifuged (*Sigma*) at 15,000 rpm for 3 minutes at room temperature. The cDNA was then eluted off the columns with the addition of 30 µl of RNase free H_2O and a 3 minute centrifugation (*Sigma*) step at 15,000 rpm. This elution step was repeated after which the samples were dried completely using a Speedy Vac (*Quantum*).

Coupling of cDNA to Cy3 and Cy5 Dyes

Initially 2.8 µl of each of the Cy3 and Cy5 dyes (1 mg/tube) (*Amersham Pharmacia*) were dried down and stored at 4°C till needed in separate tubes. The dried cDNA samples were resuspended in 9 µl of 0.1 M NaBicarbonate buffer pH 9. The cDNA was then mixed with a dried Cy dye and incubated at room temperature for 1 hour in the dark. These steps were carried out in dark conditions with the use of foil covered racks.

Labeling of Cy-coupled cDNA

The coupling reaction was stopped by the addition of 4.5 µl of 4 M hydroxylamine (*Sigma*) and incubated for a further 15 minutes at room temperature in the dark. MicroSpin columns (*Amersham Pharmacia*) were initially vortexed after which the ends were snapped off and then placed into

fresh 2 ml tubes and centrifuged at room temperature at 735 g (3000 rpm Sigma centrifuge). Columns were then placed in new 1.5 ml screw cap tubes. The Cy dye/cDNA mixes were pooled and loaded on to the centre of the columns and centrifuged (*Sigma*) at 735 g. The eluate was collected and concentrated to 17µl. Blocking agents were then added including 1 µl Cot1 DNA (1 µg/µl) (*GibcoBRL*), 3 µl oligo dA (1 µg/µl) (*Amersham Pharmacia*) and 3 µl yeast tRNA (10 µg/µl) (*Sigma*), as well as 5.1 µl of 20x SSC and 0.9 µl 10% SDS. The reactions were incubated at 95°C for 1 minute, cooled to room temperature then loaded onto a microarray slide.

2.3.4.3 Microarray Hybridisation and Washes

Hybridisations were either carried out using one chip with a special slide chamber or by a sandwich method. Figure 2.1 demonstrates the difference between the two. Slides were hybridised at 65°C in humidified conditions overnight in darkness. Slides were placed in 0.1x SSC/0.1% SDS to remove coverslips or to pull the slides apart (sandwich hybridisation) then put into slide holders which were in the first wash. The washes were then completed under darkened conditions with 1 wash in 2x SSC/0.3% SDS for 5 minutes with agitation, 1 wash in 0.1x SSC/0.1% SDS for 20 minutes with agitation and finally 0.1x SSC for 10-30 minutes with agitation. Slides were then placed in 50 ml tubes (*Becton Dickinson*) and centrifuged (*Heraeus Instruments*) to remove all liquid. The dried slides were then placed in dark boxes for scanning by a GMS 418 Array Scanner (*Genetic MicroSystems*).

2.3.4.4 Gene Validation by Real Time PCR

From the microarray analysis, genes of interest were validated by Real Time PCR. The PCR reactions were carried out in 10 µl volumes consisting of 4.2 µl dH_2O, 0.8 µl $MgCl_2$ (25 mM) (*Roche*), 1 µl sense primer (10 pM/µl) (*Proligo*), 1 µl antisense primer (10 pM/µl) (*Proligo*), 1 µl 10x LightCycler Fast Start SYBER Green enzyme (*Roche*) and 2 µl DNA template (refer to 2.2.1.1). A negative control was set up with dH_2O in place of DNA template. All reactions were

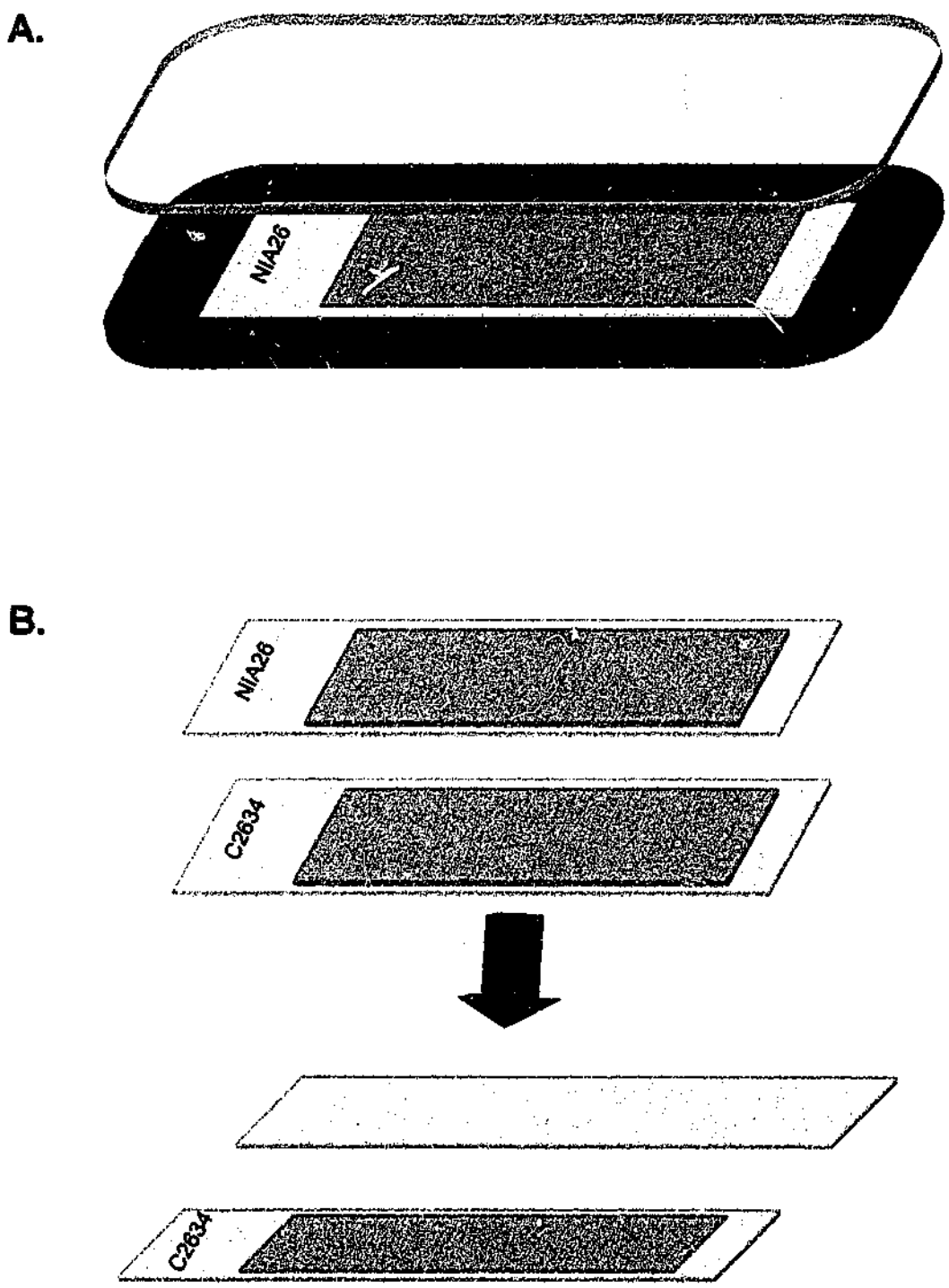


Figure 2.1: Microarray Hybridisation Methods.

A. Single microarray slides are hybridised in special humidified single slide chambers.

B. Labelled cDNA is sandwiched between two microarray slides in a sandwich hybridisation.

carried out in capillary tubes (*Roche*), that required pulse spinning (*Sigma*) at 3000 rpm to move the reaction to the bottom of the capillary tubes. The experimental protocol programed into the computer software (*Roche Molecular Biochemicals LightCycler Software Version 3.5*) of the Real Time PCR machine (*LightCycler 3 Roche*) consisted of four steps. Denaturing was the first with 1 cycle at 95°C for 30 seconds. The second step, amplification, consisted of 40 cycles at 95°C for 15 seconds, 57°C for 5 seconds (temperature values according to the melting temperatures of the primers being used), 72°C for 8 seconds (time varied according to fragment size (bp) / 25). The melting curve was the third step, consisting of one cycle of 95°C for 0 seconds, 65°C for 15 seconds and 95°C for 0 seconds. Finally the fourth step was cooling which consisted of one cycle at 40°C for 30 seconds. Standard curves were generated using concentrations of plasmid varying from 10 ng to 10^{-5} ng in order to determine concentrations of PCR products.

2.4 PROTEIN MANIPULATIONS

2.4.1 Immunoprecipitation

The spleen and lymph nodes from wild-type, *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice were harvested from the mice and made into single cell suspensions in 10% FBS DMEM. Cells were then counted and placed into 2-5 x 10^7 cell aliquots per sample to be immunoprecipitated. Cells were treated with 1000 IU/ml of muIFNα4 for 0, 10, 30 60, 120 and 240 minutes with a 10 minute pulse of muIFNα4 after which they were immediately transferred to ice and washed twice with cold PBS ensuring that all liquid was removed. Cells were then lysed with the addition of 1 ml of KALB lysis buffer pH 7.4 (Appendix A). Cells were left on ice for 15 minutes or until cells had lysed. Cell lysates were then transferred to sterile eppendorfs and centifuged (*Sigma*) at 13,000 rpm at 4°C for 10 minutes to pellet the nuclei and cell membranes. Supernatants were transferred to sterile eppendorfs to which 50 µl of protein G-sepharose (50% slurry with PBS) (*Amersham Pharmacia*) was added and rotated at 4°C for 1

hour to preclear any nonspecific binding. The sepharose was pelleted and supernatant transferred again to sterile eppendorf tubes to which 5-6 µg of STAT1 antibody (*NEB*) was added and rotated at 4°C overnight. The following day, 30 µl of protein G-sepharose (*Amersham Pharmacia*) was added and the tubes rotated for a further 30-60 minutes at 4°C. Samples were briefly spun and the supernatant discarded. The pellet was then washed 3 times with 1% Triton/PBS/1 mM vanadate in a 1 ml volume. Pellets were finally resuspended in 20 µl of 2x sample buffer.

2.4.2 Western Blot Analysis

8-12% SDS-PAGE gels were poured using 30% acrylamide (*BioRad*), H_2O, 1.5 M Tris pH 8.8, 10% SDS, ammonium persulfate (*BioRad*) and TEMED (*BioRad*) for the lower gel and 30% acrylamide, H_2O, 0.5 M Tris pH 6.8, 10% SDS, ammonium persulfate and TEMED for the 5% stacking gel. SDS-PAGE gels were electrophoresed using Hoeffer tanks (*Amersham Pharmacia*) in 1 x SDS-PAGE running buffer (Appendix A). Samples were boiled for 5 minutes and briefly spun, after which 10 µl was loaded into the wells as well as 5 µl of protein markers (*Invitrogen*). Gels were then electrophoresed at 80 volts for the samples to run through the stacking gel, then the voltage was increased to 100 volts for 1-2 hours or until the dye front had reached the bottom of the gels. The separated proteins were then transferred onto Immobilon membranes (*Millipore*) using a semi-dry transfer apparatus (*Hoefer SemiPhor™*). Immobilon membranes were first pretreated by placing in 100% methanol for 15 seconds then into dH_2O for 2 minutes and finally into transfer buffer (1 x SDS-PAGE running buffer plus 20% methanol) for at least 5 minutes. Semi-dry transfer was then assembled using 6 pieces of filter paper, the gels and the Immobilon membranes. Three pieces of filter paper soaked in the transfer buffer were placed into the apparatus, followed by the membranes, the gels and then the 3 remaining pieces of filter paper, ensuring all air bubbles were removed. The apparatus was then closed and 19 volts passed through for 40-60 minutes. After the transfer was completed, the Immobilon membranes were treated in 100% methanol for 10 seconds then air dried for 20 minutes. Filters were then

blocked using a 1% BSA/0.05% Tween/1 mM NaF/1 mM vanadate/PBS solution for 40-60 minutes at room temperature. Primary antibody was added to 1% BSA/0.05% Tween/1 mM NaF/1 mM vanadate/PBS in the appropriate dilutions (STAT1 1:200; Phosphorylated STAT1 1:200). Filters were incubated with primary antibody overnight at 4°C with agitation.

Filters were washed four times for 15 minutes each in PBS/0.05% Tween/0.1 mM vanadate/1 mM NaF with agitation. Secondary antibodies (*DAKO*) were added to 1% BSA/0.05% Tween/1 mM NaF/1 mM vanadate/PBS at 1:1000 dilution. Filters were incubated with secondary antibody for 1 hour at room temperature with agitation. Again filters were washed four times for 15 minutes each in PBS/0.05% Tween/0.1 mM vanadate/1 mM NaF. On completion of washes, filters were placed in SuperSignal Chemiluminescent substrate (1:1) (*Pierce*) for 5 minutes and subsequently exposed to X-ray film (*Kodak*) for varying times for the bands to become visible.

2.4.3 Bradford Protein Assays

Bradford protein assays were used to determine the amount of protein in each 2'-5' OAS enzyme assay lysate (refer to section 2.7.1). Various concentrations of BSA (1, 2, 4, 8, 10 and 20 µg/µl) were diluted in order to generate a standard curve. The samples were tested in duplicate with both 2 µl and 4 µl of protein lysate being assayed. Each sample was diluted into 800 µl of dH_2O and then 200 µl of Bradford reagent (*BioRad*) was added. Samples were mixed, then 200 µl of each sample transferred into a 96 well microtitre plate (*Sarstedt*) and the absorbance read using a microplate reader (*BioRad*) at a wavelength 595 nm. The readout of the assay produced the amount of protein per sample as µg/µl.

2.5 CELL CULTURING AND BIOLOGICAL ASSAYS

2.5.1 Cell Culturing

Murine L929 fibroblast cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (*Gibco*) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (*JRH Biosciences*), penicillin (50 units/ml) (*Gibco*) and streptomycin (50 µg/ml) (*Gibco*) and maintained at 37°C and 5% CO_2 in humidified air. Human 2ftgh fibrosarcoma cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (*Gibco*) supplemented with 10% (v/v) heat inactivated FBS, penicillin (50 units/ml) and streptomycin (50 µg/ml). Cell lines were passaged by initially removing culture medium, rinsing cells with PBS, incubation with 1-2 ml 0.25% trypsin/EDTA (Appendix A) and resuspended in fresh media to inactivate the trypisn/EDTA after cells were no longer adhering to the plates. After pelleting in a centrifuge (*Sigma*) at 1000 rpm, the cells were resuspended in fresh media at the appropriate dilution.

2.5.2 Bone Marrow Macrophage Culturing

To culture bone marrow derived macrophages, primary cells were flushed from the femurs of wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ 3-4 month old mice. Femurs were harvested from the mice and placed into sterile PBS. From the PBS, bones were wiped down with Kimwipe tissues to remove the muscle from around the bone. They were then placed in 70% EtOH to rinse, then placed into PBS and finally into RPMI media containing 10% FBS, 0.05% penicillin/streptomycin. The ends of the bones were cut with a scapel balde to expose the bone marrow that was then flushed with supplemented media using an 18 gauge needle. The resulting flushed bone marrow was then made into a single cell suspension. Cells were centrifuged (*Sigma*) at 1000 rpm for 10 minutes. The cell pellets were resuspended and plated out at half a femur per plate into non-adherent 10 cm^2 dishes (*Sarstedt*) in RPMI supplemented with CSF-1. Cells were cultured for 5 days after which an extra 10 ml of supplemented RPMI media was added.

On the 6th day of culturing, cells were passaged and reseeded at 1 x 10^7 cells/plate (*Becton Dickinson*). Cells were cultured overnight and experiments performed on day 7 of culture.

2.5.3 Generation and Culturing of Primary Murine Embryo Fibroblasts

Murine embryo fibroblasts were derived from day 13-14 embryos. *Socs1*$^{+/-}$ pregnant females were killed and embryos dissected out of the uterine horns and individually separated into fresh PBS as they were possibly of different genotype. Once separated the eyes and all visible internal organs were removed and remaining parts of the embryo were placed in 1-2 drops of PBS. Embryos were cut up, 1-2 ml of 0.25% trypsin/EDTA (Appendix A) added and placed at 37°C, 5% CO_2 for three 10 minute incubations to make single cell suspensions. At the end of each incubation, cells were mixed and DNase treated (16 µg) on the final two incubations. At the end of the final incubation, the cell suspensions were made up to 10 ml with DMEM supplemented with 10% (v/v) FBS and 0.5% (v/v) penicillin/streptomycin. Cells were then pelleted at 1000 rpm for 10 minutes, the supernatant discarded, resuspended in fresh supplemented DMEM and plated out into 10 cm^2 dishes (*Becton Dickinson*). These cells were passaged as mentioned in section 2.5.1 as per passaging L929 and 2ftgh cells.

2.5.4 Interferon Treatment

Cells to be harvested for poly (A)+ RNA extraction (L929 and 2ftgh cells) were passaged in 175 cm^2 flasks (*Becton Dickinson*) and grown to 80-90% confluency prior to treatment with IFN. An appropriate time course was determined, then IFN was added at 1000 IU/ml in duplicate flasks, and the cells harvested at the various time points prior to RNA extraction.

Cells to be harvested for total RNA extraction (BMMs) were treated with mulFNα4 at day 7 after extraction from femurs with 1000 IU/ml. Four hours after treatment cells were harvested along with the untreated cells as a control.

2.5.5 Preparation of Cell Pellets for Poly (A)+ and Total RNA Extraction

Cells for poly (A)+ RNA extraction were harvested by discarding media and rinsing cells with PBS. The cells were then incubated with trypsin/EDTA to detach, after which they were resuspended in 10 ml of fresh media. Cells were then centrifuged at 1000 rpm for 5 minutes, pellets washed in PBS, re-pelleted, the supernatant discarded and the cell pellets snap frozen in liquid nitrogen and stored at -80°C.

Plates that contained the cells for total RNA extraction (BMMs) were placed on ice then the media was aspirated and discarded. To each plate 1 ml of cold PBS was added and the cells were detached using a plastic disposable cell scraper (*Becton Dickinson*). An additional 1 ml of cold PBS was added to the plate and cells were transferred to a pre-chilled tube. Cells were pelleted at 1000 rpm for 3 minutes at 4°C and supernatant discarded. The pellet was then resuspended in 4 ml of RLT buffer (lysis buffer) (*Qiagen*) and homogenised by passing through an 18 gauge needle at least 10 times till the solution was no longer viscous. The samples were stored at -20°C short term or alternatively -80°C for long term.

2.5.6 Viral Infection Experiments

2.5.6.1 Organ Viral Assays (CPE Assays)

Individual frozen organs from neonatal pups were weighed then transferred to 1 ml of cold PBS on ice. Samples were then sonicated using an Ultrasonics Homogeniser set on 40 amplitude for three 5 second pulses ensuring the

sample stayed cold. Organ homogenates were centrifuged at (*Sigma*) 13,000 rpm at 4°C for 30 minutes. Supernatants were serially titrated in duplicate across two 96 flat bottom tissue culture plates (*Becton Dickinson*) in half logs. Virus was titrated onto plates as a positive control and as a negative control normal cells were left to culture for the duration of the assay. The plates contained murine L929 cells that had been plated out at a concentration of 1.3 x 10^5 cells/ml in RPMI supplemented with 3% (v/v) FBS and 0.5% (v/v) penicillin/streptomycin. Plates were then left at 37°C, 5% CO_2 for 3 days. After this time, plates were viewed under a microscope with each well being scored for death giving a score of 4 for 100% death, 3 for 75% death, 2 for 50% death and 1 for 25% death and 0 for 0% death. The viral titres were then deciphered from the scores as where 50% death occurred.

Likewise, the *Socs1* MEFs were plated out at 1.3 x 10^5 cells/ml into 96 well flat bottomed tissue culture plates (*Becton Dickinson*) in DMEM supplemented media. Virus was then added to the plates serially diluting in half logs. Plates were incubated at 37°C 5% CO_2 for 3 days, after which plates were examined under microscope and scored as described above.

The viral titres between each sample group were then subjected to statistical analysis (Unpaired t test).

2.5.6.2 Serum Interferon Assays (CPE Reduction Assays)

Serum samples collected from neonates were serially titrated in duplicate into 96 flat bottom well tissue culture plates (*Becton Dickinson*) in half logs. Samples were cultured at 37°C, 5% CO_2 overnight on murine L929 cells that had been plated into 96 flat bottomed well tissue culture plates (*Becton Dickinson*) at a concentration of 1.3 x 10^5 cells/ml in RPMI supplemented with 3% (v/v) FBS and 0.5% (v/v) penicillin/streptomycin. As a positive control, mulFNα4 obtained from Dirk Gewert (present address: BioLauncher Ltd Cambridge) as a gift was serially diluted in half logs at the same time as the serum samples were titrated. Media was removed 24 hours later and virus was

added in fresh supplemented RPMI at a dose corresponding to the TCID-50 (a dose that kills 50% of cells). Plates were incubated at 37°C, 5% CO_2 for 3 days after which plates were viewed as described in 2.5.6.1.

In order to measure the responsiveness of SOCS1, SOCS2, SOCS3, CIS overexpressing cell lines and *Socs1* MEFs to the antiviral effects of IFN they were used as target cells in a CPE reduction assay. Cells were plated into 96 flat bottom well tissue culture plates (*Becton Dickinson*) at 1.3 x 10^5 cells/ml in DMEM supplemented with 3% (v/v) FBS and 0.5% (v/v) penicillin/streptomycin. IFN was then serially diluted in half logs. Media was removed 24 hours later and virus added at the TCID-50 in fresh supplemented DMEM. Plates were incubated at 37°C, 5% CO_2 for 3 days after which plates were viewed and scored as described in 2.5.6.1.

2.5.7 MTT Assays

Human 2ftgh cell lines overexpressing SOCS1, SOCS2, SOCS3 and CIS were subject to CPE reduction assays (refer to section 2.5.6.2). To measure cell viability, cells were stained by the addition of 50 µl of 2 mg/ml of MTT (*Sigma*) to cells and media. The cells were then incubated for 4 hours at 37°C 5% CO_2 after which media with MTT was removed. To allow for a colour change to occur, 150 µl of DMSO was added to each well after which the plates were read at 595 nm within 30 minutes.

2.6 MICE

Genomic DNA was isolated from the mouse tail sections and analysed by Southern blot to determine the genotype of the mice used (refer to section 2.2.3). Throughout this project there were four different groups of mice. The first group included wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice on a 129/Sv Balb/c mixed background housed in both conventional and specific pathogen free (SPF) conditions at the Monash Institute of Reproduction and Development. The second group were wild-type, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ littermates. They were

housed in conventional conditions at the Walter and Eliza Hall Institute of Medical Research. The third group, *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ on a 129/Sv and C57bl/6 mixed background were housed at the Walter and Eliza Hall Institute of Medical Research and then also at the Monash Institute of Reproduction and Development under SPF conditions. The last group were *Socs1/Ifnar1* and *Socs1/Ifnar2* crosses that were housed in conventional conditions at the Monash Institute of Reproduction and Development.

2.6.1 Interferon Injections of Mice

In order to examine IFNα/β signaling in the type I IFN receptor knockout mice, 3 to 4 month old wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice were injected with 100 µl of muIFNα4 at a concentration of 3.3 x 10^3 IU/g of body weight into the intraperitoneal cavity. To investigate the regulation of IFN responses 14 day old neonatal wild-type, *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ were injected with muIFNα4 at a concentration of 3.3 x 10^3 IU/g of body weight into the intraperitoneal cavity. After 4 hours, the injected mice plus non-injected mice of the same number and genotype were killed and the spleens, kidneys, lungs, livers, thymus and brains were harvested and snap frozen immediately in liquid nitrogen.

2.6.2 Virus Infection of Neonates

In order to examine resistance levels, *Socs1*$^{-/-}$, *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and wild-type neonates were subjected to viral infections at 5 days of age. They were injected with 50 µl of Semliki Forest Virus (SFV) at 10x, 30x or 100x TCID-50 into the intraperitoneal cavity, where the TCID-50 is the concentration of virus that will kill 50% of cells determined by a CPE reduction assay. Mice were then checked twice daily and their survival to virus was measured in days post infection.

In order to measure viral titres and serum IFN levels, 5-10 day old neonatal mice were injected with 50 µl of SFV at 10 x TCID-50 and monitored for 2 days. These experiments were conducted on *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and wild-type mice in one

experiment and then in a separate experiment on litters from *Socs1*$^{+/-}$ matings that contained all three genotypes in a Mendelian 1:2:1 frequency. After 48 hours mice were killed and blood, spleen, kidneys, liver, thymus, lungs and the brain harvested. The blood was left to coagulate after which it was centrifuged (*Sigma*), the serum collected and stored at 4°C. The organs harvested were snap frozen in liquid nitrogen and stored at -80°C.

2.6.3 Organ Dissection for Histology

Litters generated from *Socs1*$^{+/-}$ *Ifnar1*$^{-/-}$ matings that survived to 3-6 weeks of age were sacrificed for histology. Mice were killed and the following organs and tissues harvested and placed into 10% formalin: skin, skeletal muscle from the top of the femur, testis/ovaries, bladder, kidneys, spleen, pancreas, a segment of the small intestine, mesenteric lymph nodes, a portion of the liver, thymus, lungs, heart, salivary gland, thyroid, eyes, brain, femur and the sternum.

2.7 2'-5' OLIGOADENYLATE SYNTHETASE ASSAY (OAS)

2'-5' Oligoadenylate synthetase is a type I IFN inducible enzyme that is produced in the presence of viral double stranded RNA. The function of this enzyme is to produce oligoadenylate chains in 2'-5' structure in order to activate RNaseL which is then able to degrade the viral RNA. The more oligoadenylate chains produced the higher the activity of the 2'-5' OAS enzyme. This can be measured by incorporation of radioactive label into the oligoadenylate chains that are produced, therefore the higher the radioactive counts of the samples are, the higher the activity of the enzyme. This assay was conducted on murine embryo fibroblasts (refer to section 2.5.3) that were wild-type, *Socs1*$^{+/-}$ or *Socs1*$^{-/-}$.

2.7.1 Treatment and Harvesting of Murine Embryo Fibroblasts

Cells were cultured and passaged as outlined in section 2.5.3 and treated with muIFNα4 at 1000 IU/ml with an initial 24 hour pulse using time points of 0, 24, 48, 72, 96, 120 and 144 hours. After 24 hours the culture media was changed on the remaining plates and incubated until the appropriate time point. Cells were trypsinised and subsequently lysed at 1 x 10^6 cells/100 µl of lysis buffer (Appendix A) on ice for 10 minutes after which they were centrifuged (*Sigma*) at 13,000 rpm for 10 minutes at 4°C and supernatants collected and stored at -80°C.

2.7.2 2'-5' OAS Assay

Ten microlitres of lysates were mixed with 40 µl of 1:2 slurry with buffer C (Appendix A) of poly I:poly C agarose beads (*Amersham Pharmacia*) then incubated at 30°C for 15 minutes. The beads were washed with 1 ml of buffer C then incubated with a reaction mixture containing α-^{32}P dATP (*Amersham Pharmacia*) for 16 hours at 30°C. Alkaline phosphatase (*Roche*) was added to each reaction and incubated for 2 hours at 37°C. After this time, reactions were diluted with dH_2O and centrifuged at 13000rpm for 30 seconds (*Sigma*) and 10 µl of the supernatant was further diluted in 90 µl of buffer A (acidic) (Appendix A) and passed through an alumina gel column (*Sigma*) with 5 ml of buffer A. The eluates were collected and counted on a tritium channel on the 1900TR Liquid Scintillation β counter (*Canberra Packard*). The radioactive counts obtained were subjected to statistical analysis (Mann-Whitney t tests).

2.8 COMPUTER ANALYSIS PROGRAMS

Throughout this project all statistics were carried out on GraphPad Prism software, including t tests logrank tests and rank sum tests.

All microarray analysis was completed initially using Imagene and then for more advanced analysis Genespring 5.0 software.

CHAPTER 3

IFN induced SOCS1 and SOCS3 Regulate IFN Responses *in vitro*

3.1 INTRODUCTION

Signal transduction as a result of both type I and II IFN stimulation is initiated after ligand-receptor binding to elicit numerous biological effects. The JAK/STAT pathway is the most comprehensively studied signaling pathway utilised by the IFNs (Darnell *et al.*, 1994). Both subunits of the type I IFN receptor, IFNAR1 and IFNAR2 lack intrinsic kinase activity and are pre-associated with TYK2 and JAK1 respectively (Stark & Kerr, 1992; Richter *et al.*, 1998; Kotenko *et al.*, 1999). After IFN binds to the receptor the JAKs undergo a trans-phosphorylation resulting in both JAK1 and TYK2, and subsequently the receptor chains being tyrosine phosphorylated. This enables latent STAT1 and 2 proteins to dock onto the phosphorylated receptor and subsequently become tyrosine phosphorylated. The STATs then dissociate from the receptor unit, as their affinity for each other is stronger than for the phosphorylated receptor complex (Schindler & Darnell, 1995; Bluyssen *et al.*, 1996). The STAT1/STAT2 heterodimer then binds IRF9 and translocates to the nucleus as the ISGF3 transcriptional activation complex (Schindler & Darnell, 1995). The activation of the JAK/STAT pathway is very rapid and transient and has been demonstrated to occur within minutes of stimulation lasting for 30-60 minutes, even though the biological effects that they initiate may be observed hours later (Schindler *et al.*, 1992; Leonard & O'Shea, 1998).

One of the definitive biological effects of IFNs is its ability to protect from acute viral infection. However there are adverse effects of too much IFN that can result in toxicity (Gutterman *et al.*, 1994). Therefore mechanisms have evolved to achieve a balance between positive and negative regulation enabling signaling pathways to be rapidly activated and then deactivated to achieve the beneficial effects without the harmful side effects. How this regulation is achieved is the focus of this project. There are several negative regulators of signaling that are able to act at different levels of signal transduction. As reviewed in chapter 1 these include soluble receptors (e.g. IFNAR2a) (Kim & Maniatis, 1996; Strous *et al.*, 1996; Hardy *et al.*, 2001), protein tyrosine phosphatases (e.g. SHP1) (Neel, 1997; Irie-Sasaki *et al.*, 2001), inhibitors of activated STAT (e.g. PIAS1) (Chung *et al.*, 1997; Neel, 1997; Irie-Sasaki *et al.*,

2001) and the suppressor of cytokine signaling proteins (e.g. SOCS1).(Starr *et al.*, 1997; Endo *et al.*, 1997; Naka *et al.*, 1997).

At the commencement of these studies, the SOCS family of proteins had just been discovered and charaterised as negative regulators of cytokine signaling with the ability to inhibit the JAK/STAT pathway by different mechanisms. SOCS1 was discovered simultaneously by three independent groups as an inhibitor of IL6 signaling (Starr *et al.*, 1997), as a JAK binding protein (Endo *et al.*, 1997) and as a STAT induced STAT inhibitor (Naka et al., 1997). Studies since have demonstrated SOCS1 directly interacts with JAK1, JAK2 and TYK2 blocking their phosphorylation resulting in a loss of JAK kinase activity and subsequent signal transduction (Nicholson *et al.*, 1999; Yasukawa *et al.*, 1999). Subsequently, based on sequence similarity to two conserved regions (the SH2 domain and the C-terminal SOCS box) of *Socs1*, the remaining SOCS family members were identified in the GenBank databases and named *Socs2-7* sequentially (Starr *et al.*, 1997). Unlike SOCS1, SOCS3 acts by binding to phosphorylated receptors blocking further binding by cytoplasmic transcription factors such as STATs stopping further signal transduction (Schmitz *et al.*, 2000; Bjorbak *et al.*, 2000). *Cis* was discovered before *Socs1* as an immediate early gene containing a SH2 domain induced by a range of cytokines (Yoshimura *et al.*, 1995). After the discovery of *Socs1*, *Cis* was found to also contain a SOCS box domain making it a member of the SOCS family of proteins. *In vitro* and *in vivo* data suggest that CIS is able to compete for the same binding site as STAT5 on the activated IL2, IL3 and EPO receptors (Yasukawa *et al.*, 2000). CIS has been proposed to function as an adapter protein between activated cytokine receptors binding to phosphorylated tyrosines and other cytoplasmic negative regulators (Matsumoto *et al.*, 1999).

At the initiation of this study the effect of IFNs on *Socs* gene expression was unknown. Therefore the *in vitro* effect on the gene expression of *Socs1*, *2*, *3* and *Cis* transcripts being the first four family members available for experimentation, by both type I and II IFNs has been examined. Both *Socs1* and *Socs3* were selectively induced by both type I and II IFNs, therefore in order to examine the effect these genes/proteins had on IFN signaling was

subsequently examined. The data shows that not only are *Socs1* and *Socs3* selectively induced by type I and II IFNs, but they are also potent inhibitors of IFNs antiviral activity.

3.2 RESULTS

3.2.1 Type I and II IFNs Selectively Induce *Socs1* and *Socs3* Gene Expression

Firstly, we examined whether IFNs, which use the JAK/STAT pathway were capable of regulating the expression of SOCS proteins. Murine L929 and human 2ftgh cells were used, as they are highly responsive to IFNs (Domanski *et al.*, 1995; Lutfalla *et al.*, 1995). Both human and mouse cells were treated with 1000 IU/ml of IFNα4, IFNβ and IFNγ for 0, 0.25, 0.5, 1, 2, 3, 4, 6, 12 and 24 hours. Poly (A+) RNA was extracted, electrophoresed through 1% denaturing gels, transferred to Hybond C extra membranes and subsequently hybridised with ^{32}P-labeled cDNA fragments for *Socs1*, *Socs2*, *Socs3* and *Cis* (refer to 2.2.2).

3.2.1.1 Socs1

The 1.4 kb *Socs1* transcript was induced by IFNα4, IFNβ as well as IFNγ in both L929 cells (Figure 3.1A and B) and 2ftgh cells (Figure 3.2A). Each of the IFNs induced the *Socs1* transcript with different kinetics. When induction levels from L929 cells were normalised to *Gapdh* levels and expressed as fold induction IFNα4 induced *Socs1* 5-fold within 30 minutes then by 4 hours post treatment this increased to a 7-fold induction (Figure 3.1A). In contrast IFNβ induced *Socs1* expression 7-fold within 15 minutes, peaking at 60 minutes with a 40-fold induction, then decreasing to a 13-fold induction 4 hours after initial treatment (Figure 3.1A). IFNγ, examined over a longer time course, induced *Socs1* 5-fold within 1 hour peaking at 6 hours with a 53-fold increase, then by 24 hours post treatment had decreased to 20-fold (Figure 3.1B). When induction levels from human 2ftgh treated cells were normalised to *Gapdh* and expressed as fold induction, the 1.4 kb *Socs1* transcript was induced by IFNβ 2-fold 3 hours post treatment (Figure 3.2A), while IFNγ induced *Socs1* 2.7-fold within 3 hours of treatment (Figure 3.2A).

Figure 3.1: Induction of *Socs1* by IFNα4, IFNβ and IFNγ in Murine Cell Line L929.

Poly (A)+ RNA was extracted from IFN treated murine L929 cells and bound to Hybond C extra nitrocellulose membranes following electrophoresis. The filters were then sequentially hybridised with *Socs1* and then *Gapdh* for normalisation of mRNA levels.

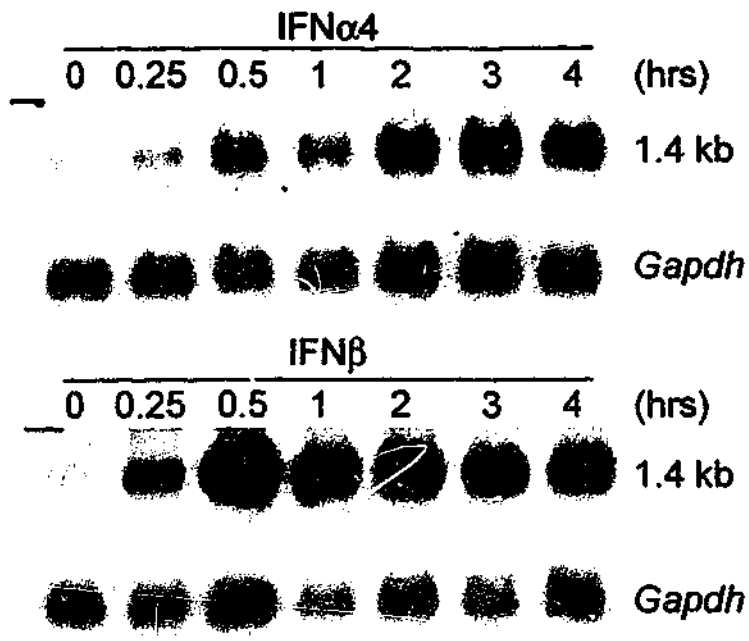
A. Northern blots of *Socs1* and *Gapdh* after treatment over a time course with IFNα4 and IFNβ.

B. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. An 8-fold induction by IFNα4 ■ after 4 hours was observed while a 41-fold induction was induced by IFNβ ■ within 1 hour of treatment.

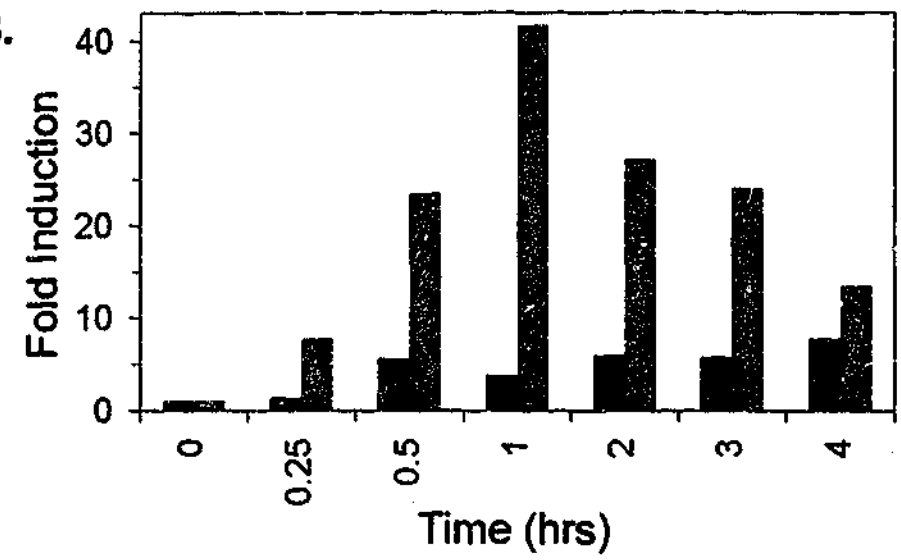
C. Northern blots of *Socs1* and *Gapdh* after treatment over a time course with IFNγ.

D. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. A 50-fold increase 6 hours post IFNγ ■ treatment was observed.

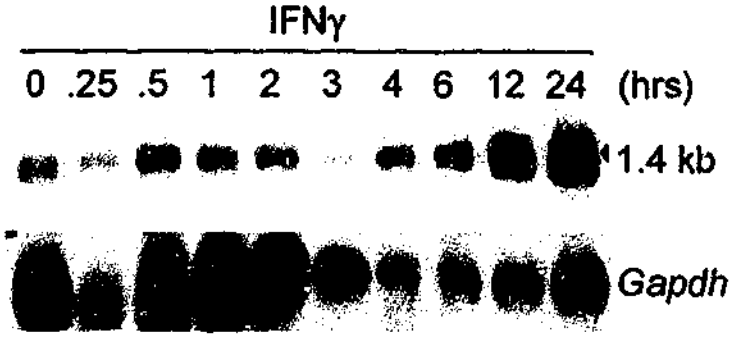
A.


IFNα4
0 0.25 0.5 1 2 3 4 (hrs)
1.4 kb
Gapdh
IFNβ
0 0.25 0.5 1 2 3 4 (hrs)
1.4 kb
Gapdh

B.


Fold Induction
0
10
20
30
40
0
0.25
0.5
1
2
3
4
Time (hrs)

C.


IFNγ
0 .25 .5 1 2 3 4 6 12 24 (hrs)
1.4 kb
Gapdh

D.

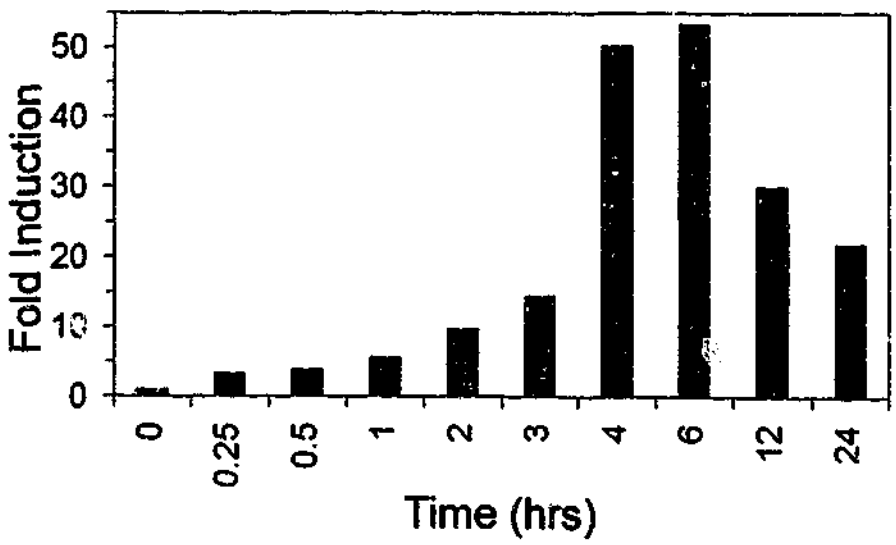

Fold Induction
0
10
20
30
40
50
0
0.25
0.5
1
2
3
4
6
12
24
Time (hrs)

Figure 3.2: Induction of *Socs1* and *Socs3* by IFNβ and IFNγ in Human Cell Line 2fgth.

Poly (A)+ RNA was extracted from IFN treated human 2ftgh cells and bound to Hybond C extra nitrocellulose membranes following electrophoresis. The filters were then sequentially hybridised with *Socs1* or *Socs3* and then *Gapdh* for normalisation of mRNA levels.

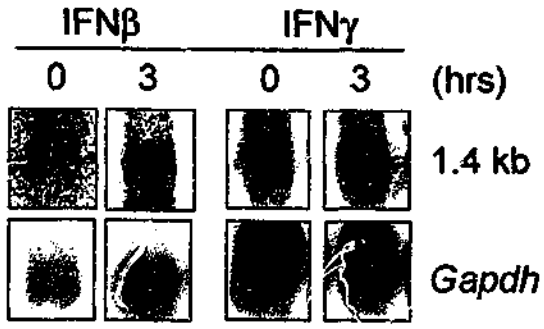
A. Northern blots of *Socs1* induced in human 2ftgh cells treated with IFNβ and IFNγ 3 hours after treatment.

B. Induction levels were normalised to *Gapdh* and expressed graphically as fold induction relative to time 0. *Socs1* was induced 2-fold and 2.7-fold by IFNβ ▩ and IFNγ ■ respectively.

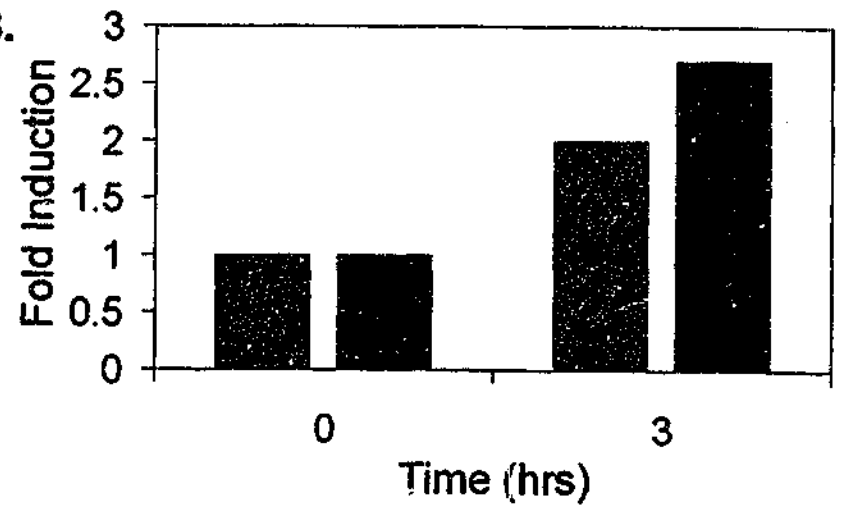
C. Northern blots of *Socs3* induced in human 2ftgh cells treated with IFNβ and IFNγ 1 hour after treatment.

D. Induction levels were normalised to *Gapdh* and expressed graphically as fold induction relative to time 0. *Socs3* was induced 3.2-fold and 3.6-fold by IFNβ ▩ and IFNγ ■ respectively.

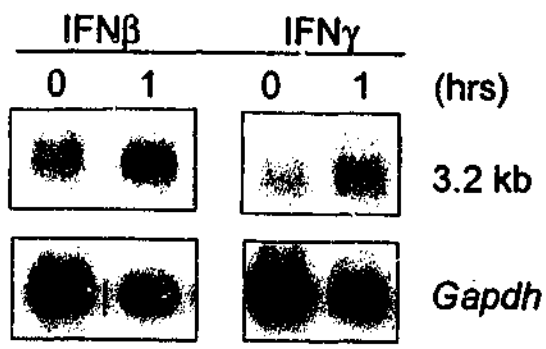
A.


IFNβ
IFNγ
0
3
0
3
(hrs)
1.4 kb
Gapdh

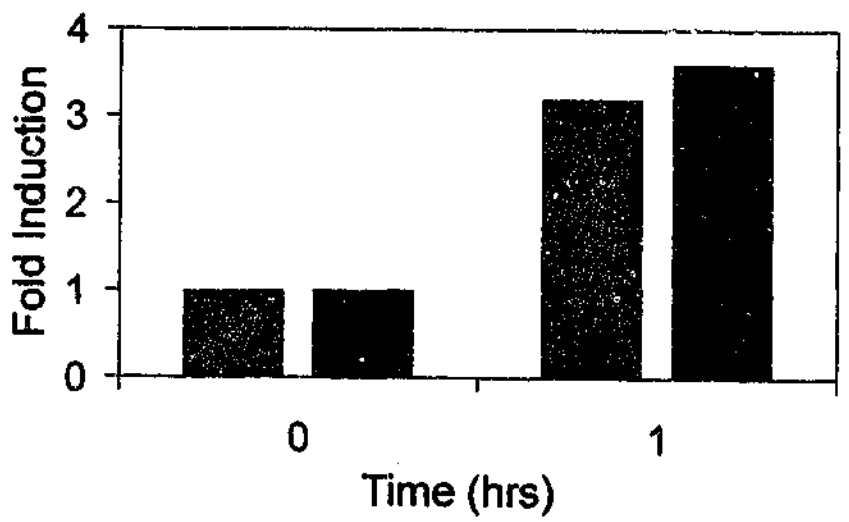
B.


Fold Induction
3
2.5
2
1.5
1
0.5
0
0
3
Time (hrs)

C.


IFNβ
IFNγ
0
1
0
1
(hrs)
3.2 kb
Gapdh

D.


Fold Induction
4
3
2
1
0
0
1
Time (hrs)

3.2.1.2 Socs3

The 3.2 kb *Socs3* transcript was induced by IFNα4, IFNβ as well as IFNγ in both murine L929 cells (Figure 3.3A and B) and human 2ftgh cells (Figure 3.2B). However, each of the IFNs induced the *Socs3* transcript with different kinetics. When induction levels from L929 cells were normalised to *Gapdh* levels and expressed as fold induction IFNα4 induced *Socs3* 3.1-fold within 30 minutes then by 4 hours post treatment had not changed significantly with a 3.4-fold induction. In contrast similar to *Socs1*, IFNβ induced *Socs3* expression 6.5-fold within 15 minutes of treatment, peaking at 30 minutes with an 11-fold increase, then decreasing back to basal levels within 2 hours (Figure 3.3A). IFNγ again examined over a longer time course, induced the 3.2 kb *Socs3* transcript later by 4.6-fold 6 hours post treatment peaking at 12 hours with a 9.8-fold induction which by 24 hours had decreased marginally to 9.5-fold (Figure 3.3B). When induction levels from human 2ftgh cells were normalised to *Gapdh* and expressed as fold induction, the 3.2 kb *Socs3* transcript was induced by IFNβ 3.2-fold 1 hour post treatment (Figure 3.2A), while IFNγ induced *Socs3* 3.6-fold within 1 hour of treatment (Figure 3.2A).

3.2.1.3 Socs2

Socs2 expression was not induced by IFNα4 in L929 cells, rather a constant basal level was maintained for the duration of the time course examined (Figure 3.4A). IFNβ induced a marginal 2.6-fold increase 2 hours after treatment that had reduced to below 2-fold 4 hours post treatment (Figure 3.4A). A 3.4-fold increase in the level of *Socs2* mRNA was observed after 6 hours of IFNγ treatment whereas at all other time points there were no changes (Figure 3.4B).

3.2.1.4 Cis

Results of the Northern hybridisations with *Cis* were quite different to that of *Socs1-3*, in that two transcripts were detected each being differentially induced by IFNβ and IFNγ (Figure 3.5). In murine L929 cells after induction levels had been normalised to *Gapdh*, IFNβ treatment induced the expected 2.5 kb *Cis*

Figure 3.3: Induction of *Socs3* by IFNα4, IFNβ and IFNγ in Murine Cell Line L929.

Poly (A)+ RNA was extracted from IFN treated murine L929 cells and bound to Hybond C extra nitrocellulose membranes following electrophoresis. The filters were then sequentially hybridised with *Socs3* and then *Gapdh* for normalisation of mRNA levels.

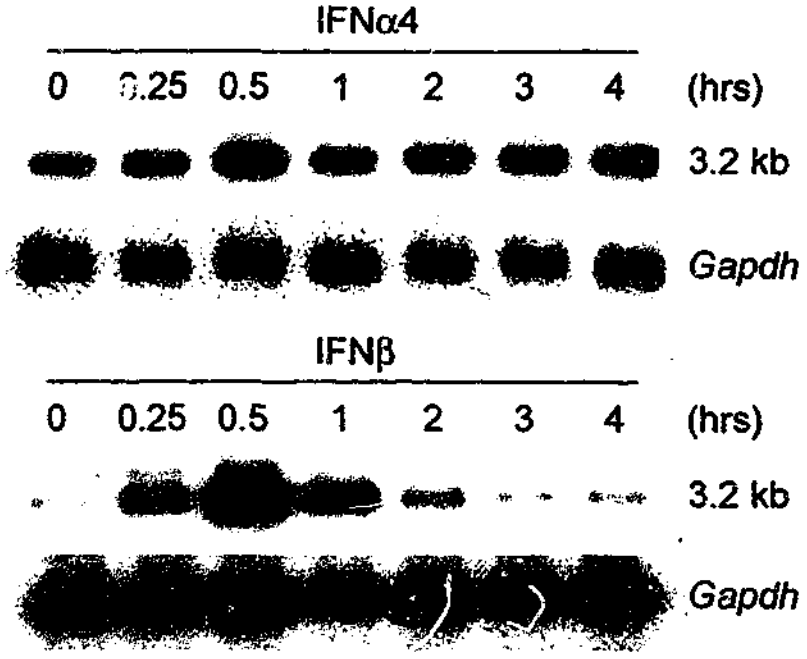
A. Northern blots of *Socs3* and *Gapdh* after treatment over a time course with IFNα4 and IFNβ.

B. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. A 3.4-fold induction by IFNα4 ■ after 4 hours was observed while a 12-fold induction was induced by IFNβ ■ within 30 minutes of treatment.

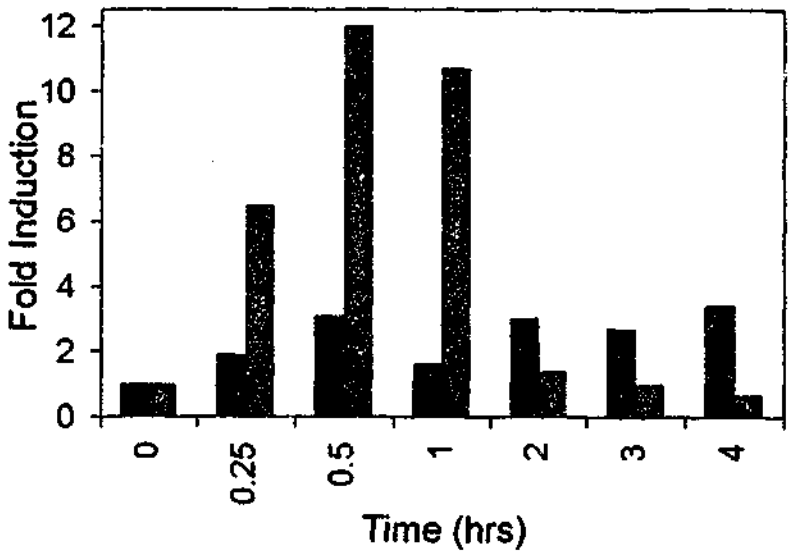
C. Northern blots of *Socs3* and *Gapdh* after treatment over a time course with IFNγ.

D. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. A 9.8-fold induction 6 hours post IFNγ ■ treatment was observed.

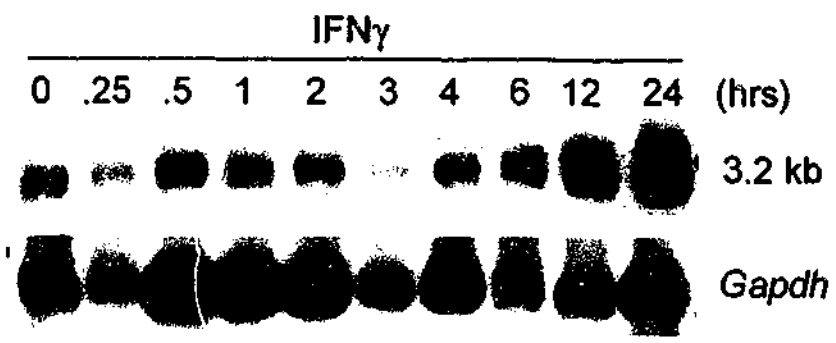
A.


IFNα4
0 0.25 0.5 1 2 3 4 (hrs)
3.2 kb
Gapdh
IFNβ
0 0.25 0.5 1 2 3 4 (hrs)
3.2 kb
Gapdh

B.


Fold Induction
0
2
4
6
8
10
12
0
0.25
0.5
1
2
3
4
Time (hrs)

C.


IFNγ
0 .25 .5 1 2 3 4 6 12 24 (hrs)
3.2 kb
Gapdh

D.

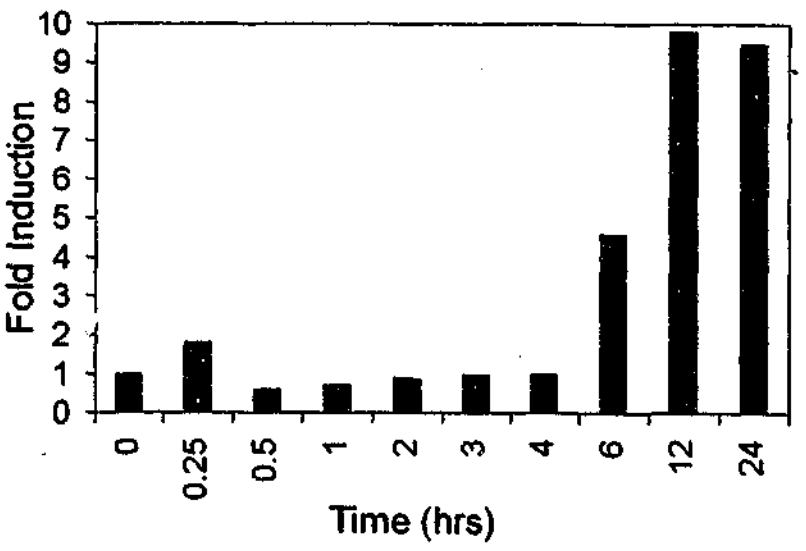

Fold Induction
0
1
2
3
4
5
6
7
8
9
10
0
0.25
0.5
1
2
3
4
6
12
24
Time (hrs)

Figure 3.4: Induction of *Socs2* by IFNα4, IFNβ and IFNγ in Murine Cell Line L929.

Poly (A)+ RNA was extracted from IFN treated murine L929 cells and bound to Hybond C extra nitrocellulose membranes following electrophoresis. The filters were then sequentially hybridised with *Socs2* and then *Gapdh* for normalisation of mRNA levels.

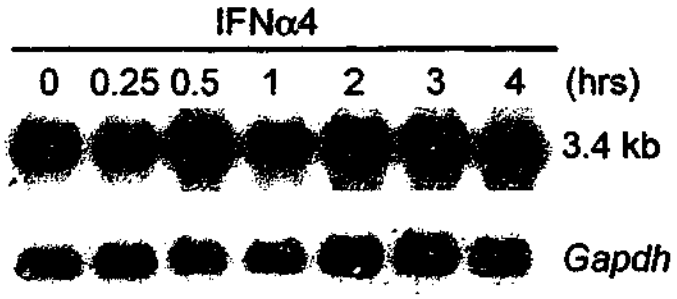
A. Northern blots of *Socs2* and *Gapdh* after treatment over a time course with IFNα4 and IFNβ.

B. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. *Socs2* was not induced by IFNα4 ■, however a 2.6-fold induction by IFNβ ■ was observed 2 hours post treatment.

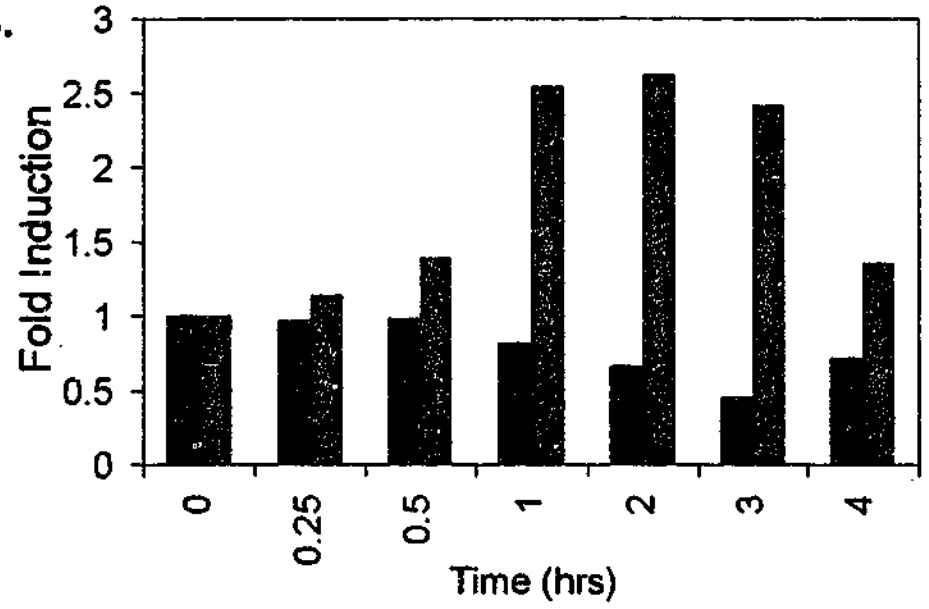
C. Northern blots of *Socs2* and *Gapdh* after treatment over a time course with IFNγ.

D. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. A 3.4-fold increase 6 hours post IFNγ ■ treatment was observed.

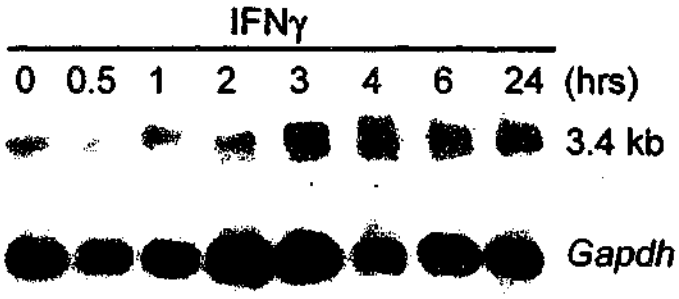
A.


IFNα4
0 0.25 0.5 1 2 3 4 (hrs)
3.4 kb
Gapdh
IFNβ
0 0.25 0.5 1 2 3 4 (hrs)
3.4 kb
Gapdh

B.


Fold Induction
3
2.5
2
1.5
1
0.5
0
0
0.25
0.5
1
2
3
4
Time (hrs)

C.


IFNγ
0 0.5 1 2 3 4 6 24 (hrs)
3.4 kb
Gapdh

D.

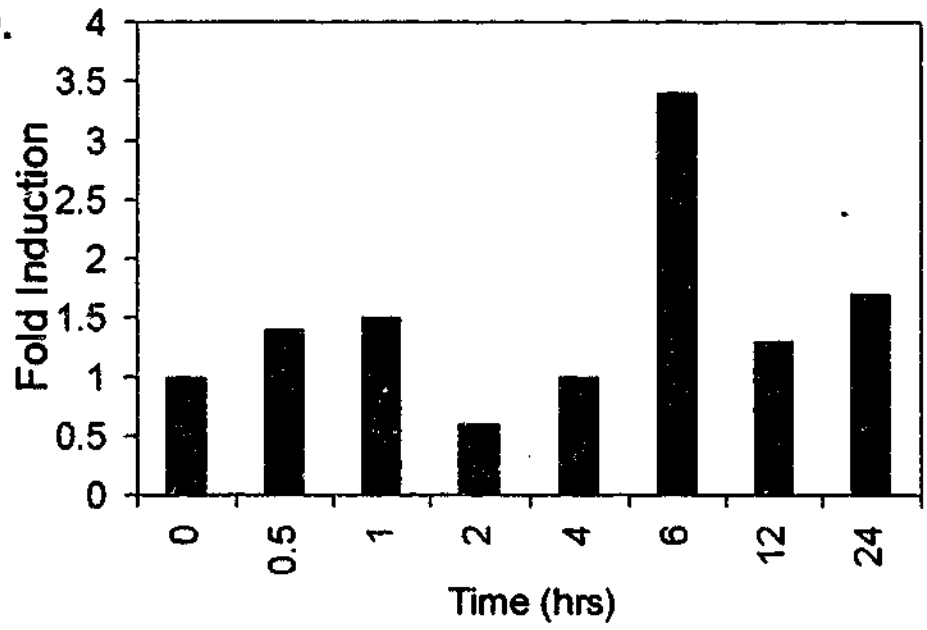

Fold Induction
4
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
1
2
4
6
12
24
Time (hrs)

Figure 3.5: Induction of *Cis* by IFNβ and IFNγ in Murine L929 Cells.

Poly (A)+ RNA was extracted from IFN treated murine L929 cells and bound to Hybond C extra nitrocellulose membranes following electrophoresis. The filters were then sequentially hybridised with *Cis* and then *Gapdh* for normalisation of mRNA levels.

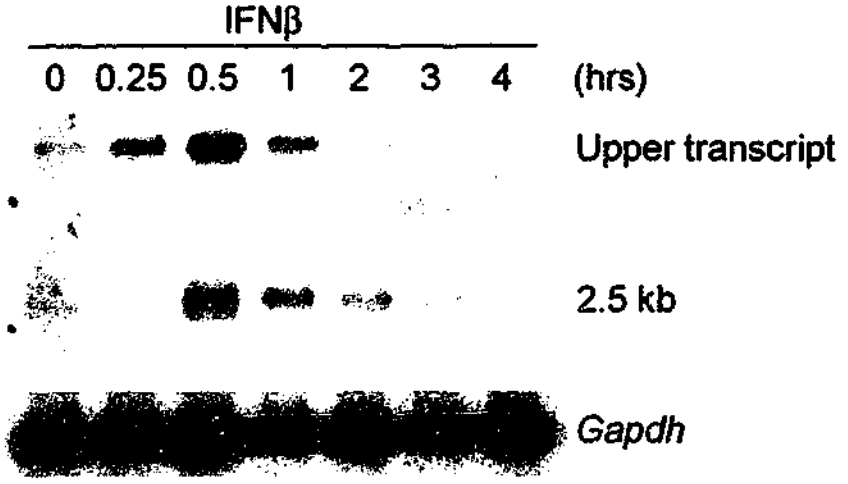
A. Northern blots of *Cis* and *Gapdh* after treatment over a time course with IFNβ. The *Cis* fragment used to hybridise to the filters generated 2 transcripts.

B. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. Treatment with IFNβ resulted in a 4-fold induction of the lower transcript ■ 30 minutes after treatment and a 3.6-fold induction of the upper transcript ■ 1 hour after treatment.

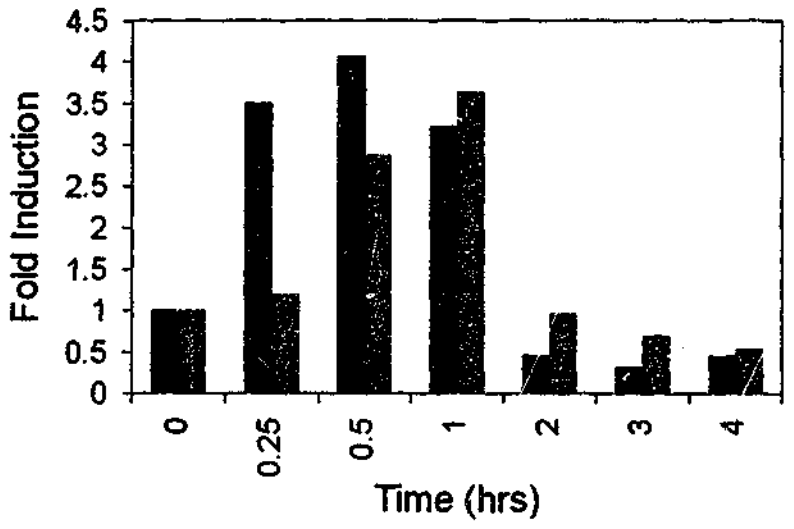
C. Northern blots of *Cis* and *Gapdh* after treatment over a time course with IFNγ. The *Cis* fragment used to hybridise to the filters generated 2 transcripts.

D. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. Treatment with IFNγ induced the upper *Cis* transcript ■ 2.1-fold after 2 hours, however the lower *Cis* transcript ■ was unchanged after treatment.

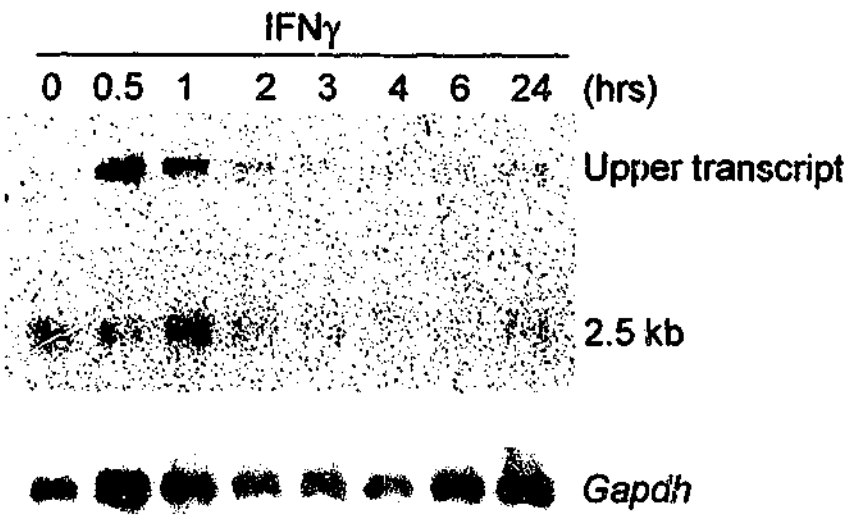
A.


IFNβ
0 0.25 0.5 1 2 3 4 (hrs)
Upper transcript
2.5 kb
Gapdh

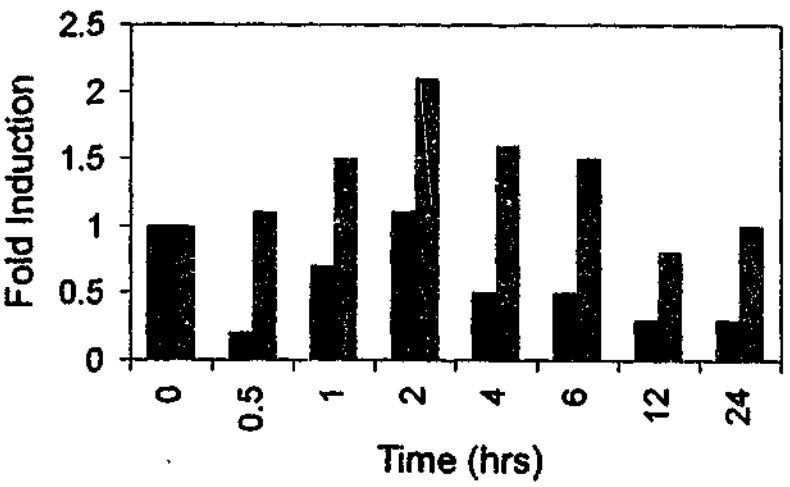
B.


Fold Induction
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
0.25
0.5
1
2
3
4
Time (hrs)

C.


IFNγ
0 0.5 1 2 3 4 6 24 (hrs)
Upper transcript
2.5 kb
Gapdh

D.


Fold Induction
2.5
2
1.5
1
0.5
0
0
0.5
1
2
4
6
12
24
Time (hrs)

transcript 2.9-fold after 30 minutes, peaking at 1 hour with a 3.6-fold induction, returning to basal levels 2 hours post initial treatment. The upper transcript (>4.4 kb) was induced 3.5-fold after 15 minutes, peaking at 30 minutes with a 4-fold increase decreasing back to basal levels by 2 hours (Figure 3.5A). When IFNγ was used to treat the L929 cells, the 2.5 kb transcript was not induced, but rather suppressed 3-fold at 30 minutes returning to basal levels by 2 hours. In contrast the upper transcript (>4.4 kb) was marginally induced 2.1-fold 2 hours after treatment decreasing back to basal levels 12 hours post initial treatment (Figure 3.5B).

3.2.2 Overexpression of SOCS1 and SOCS3 Inhibits Type I IFN Antiviral Actvity

Results from the previous section demonstrated convincingly that both type I and II IFNs were able to induce the gene expression of both *Socs1/SOCS1* and *Socs3/SOCS3*. In contrast, type I and II IFNs had relatively little effect on the gene expression of *Socs2/SOCS2* and *Cis/CIS*. Previous studies have shown that the SOCS family of proteins act in a negative regulatory capacity on cytokines that induce their expression (Yoshimura *et al.*, 1995; Starr *et al.*, 1997; Masuhara *et al.*, 1997; Naka *et al.*, 1997; Adams *et al.*, 1998; Bjoraek *et al.*, 1998; Helman *et al.*, 1998; Sakamoto *et al.*, 1998; Suzuki *et al.*, 1998). Therefore cell lines overexpressing each of the *Socs1-3* and *Cis* genes were utilised to examine whether *in vitro* they were able to regulate IFN signaling. More specifically, whether they could regulate the antiviral activity elicited by both type I and II IFNs.

Stable human 2ftgh cell lines overexpressing SOCS1 (1-4 and 1-7 represent two independent clones), SOCS2 (2-4 and 2-7), SOCS3 (3-6 and 3-9) and CIS (NC2 and NC6) were utilised in a series of antiviral bioassay experiments (refer to 2.5.6.2) to determine the antiviral potency of IFN. All overexpressing cell lines, once plated out were pre-treated with concentrations ranging from 3-10,000 IU/ml of IFNα4, IFNβ and IFNγ. Subsequently all cells were treated with SFV at 10 x TCID-50 and cultured for a further 3 days at 37°C 5% CO_2. Cells

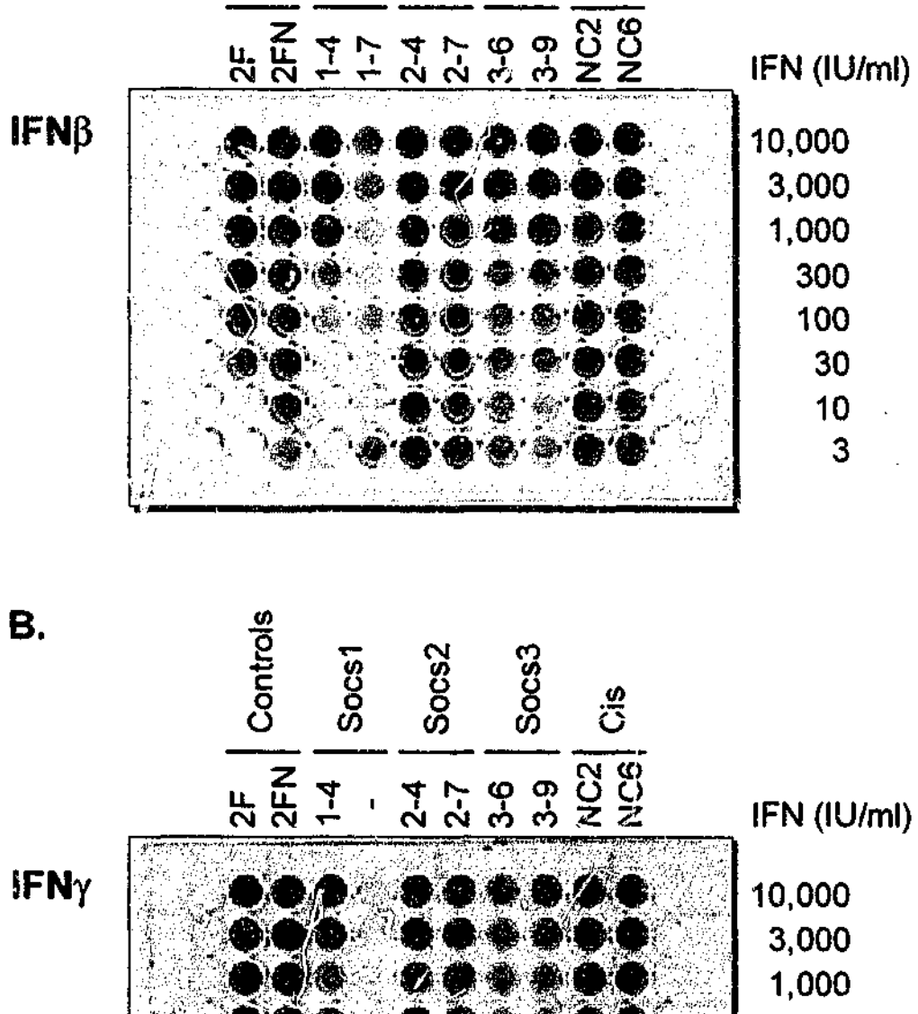
were then stained with MTT (refer to section 2.5.7) as a measure of cell viability. Live cells take up the MTT stain where as dead cells do not. Therefore coloured wells represented viable cells where the IFN had protected against the virus and colourless wells represented cells that the IFN had failed to protect against the virus and had therefore been killed (Figure 3.6). The plates shown in figure 3.6 are representative of assays performed multiple times in triplicate by Assoc. Prof. Paul Hertzog from Centre for Functional Genomics and Human Disease. Results demonstrated that both SOCS1 and SOCS3 overexpressing cell lines were less responsive to the antiviral effects of IFNs requiring 1000-3000 IU/ml of IFNβ (Figure 3.6A), IFNγ (Figure 3.6B) and IFNα (data not shown). In contrast both SOCS2 and CIS overexpressing cell lines were equally if not more responsive to IFN than the parental 2ftgh cell lines (Figure 3.6) with as little as 3 IU/ml of IFN protecting the cells from SFV infection. It is important to note that SOCS2, SOCS3 and CIS were overexpressed more than SOCS1 (data not shown), however SOCS1 demonstrated the highest inhibition of IFN antiviral signaling making it the more potent inhibitor compared to SOCS3.

The results of all the bioassays completed on these cell lines are summarised in figure 3.7 as a percentage of antiviral activity of each IFN. Clearly both SOCS1 and SOCS3 overexpressing cell lines had significantly decreased antiviral activity in response to IFNα (Figure 3.7A), IFNβ (Figure 3.7B) and IFNγ (Figure 3.7C) in comparison to the parental 2ftgh cell lines. In contrast the cell lines overexpressing SOCS2 and CIS displayed slightly higher antiviral activities than the parental cells.

Figure 3.6: Effect of SOCS Overexpression on IFN Antiviral Activity.

Bicassay results demonstrating the antiviral potency of IFNβ **A.** and IFNγ **B.** in cell lines overexpressing SOCS1 (1-4 and 1-7 represent two independent clones), SOCS2 (2-4 and 2-7), SOCS3 (3-6 and 3-9) and CIS (NC2 and NC6). Controls are the parental 2ftgh cell (2F) or after transfection with the empty neo vector (2FN). The photograph is of cells pretreated with different concentrations of IFN ranging from 3-10000 IU/ml after which they were infected with SFV at 10 x TCID-50. Cells were then stained with MTT (refer to 2.5.7). Viable cells took up the stain and are therefore represented by the coloured wells, while cells that were killed by the virus lack colour.

A.


Controls
Socs1
Socs2
Socs3
Cis
2F
2FN
1-4
1-7
2-4
2-7
3-6
3-9
NC2
NC6
IFN (IU/ml)
IFNβ
10,000
3,000
1,000
300
100
30
10
3

B.

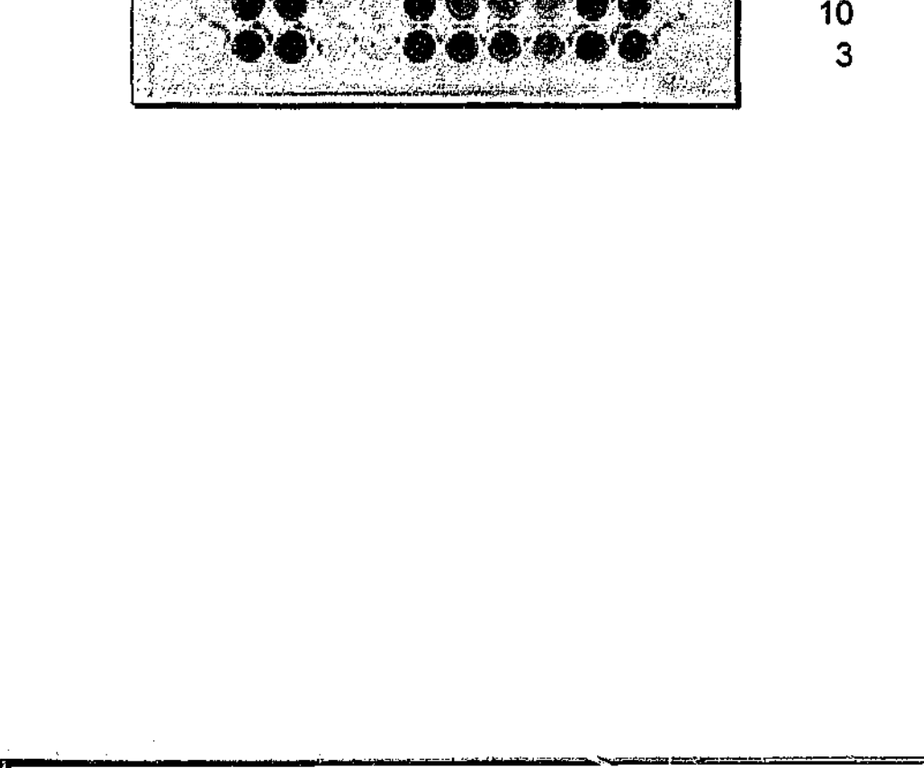
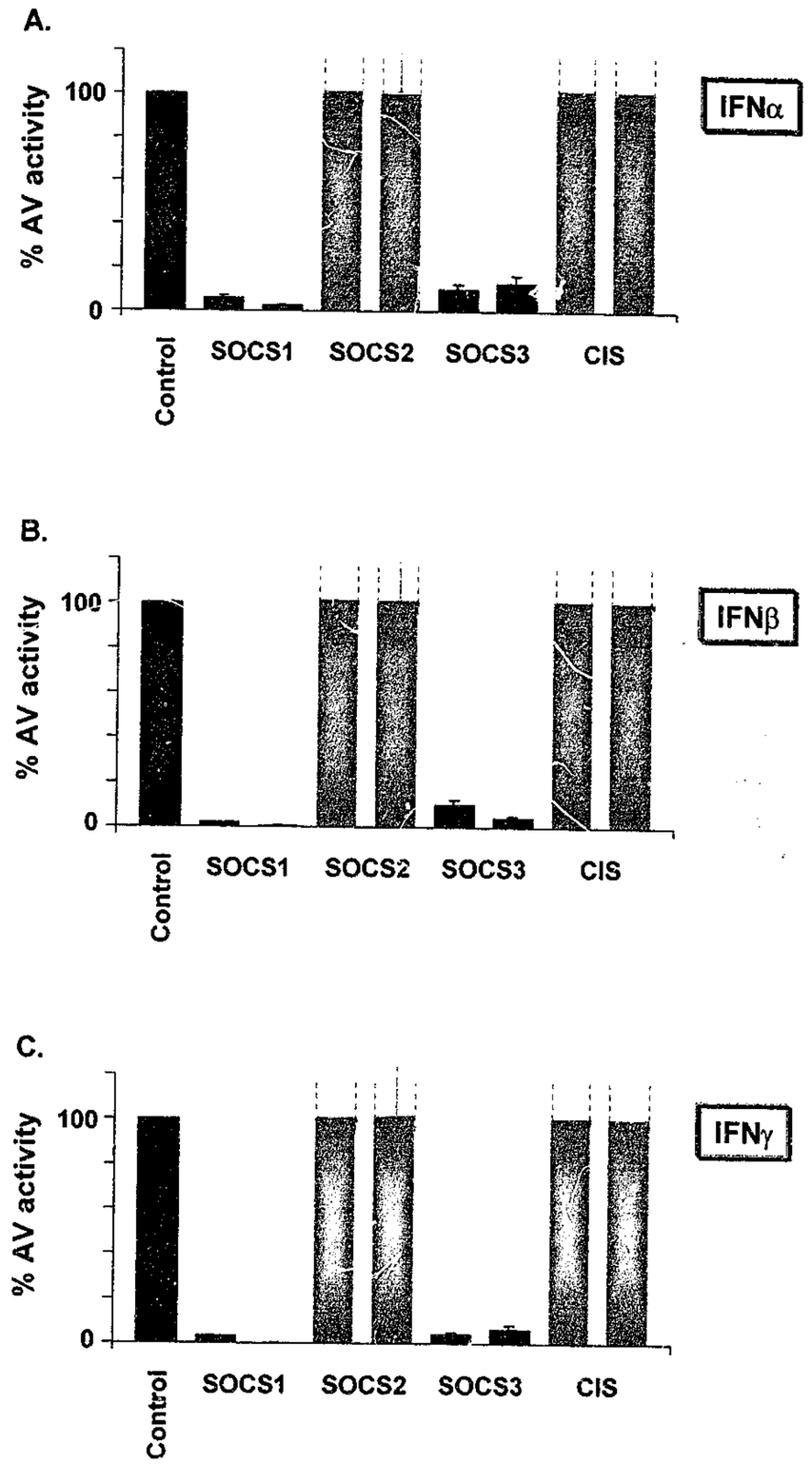

Controls
Socs1
Socs2
Socs3
Cis
2F
2FN
1-4
-
2-4
2-7
3-6
3-9
NC2
NC6
IFN (IU/ml)
IFNγ
10,000
3,000
1,000
300
100
30
10
3

Figure 3.7: Decreased Sensitivity of SOCS1 and SOCS3 Overexpressing Cell Lines to IFNs Antiviral Effects.

Bioassay results expressed as % Antiviral activity demonstrate clearly that SOCS1 ■ and SOCS3 ■ overexpressing cell lines lack protection from IFNα **A.**, IFNβ **B.** or IFNγ **C.** against viral challenge. In contrast SOCS2 □ and CIS □ display similar to higher antiviral activity than the parental control cells ■ .



3.3 DISCUSSION

Socs1 and *Socs3* but not *Socs2* or *Cis* genes have been demonstrated to be inducible by both type I and II IFNs in human and murine cells *in vitro*. Functional studies using 2ftgh cell lines stably overexpressing SOCS1-3 and CIS demonstrated that SOCS1 and 3 but not SOCS2 or CIS inhibited the antiviral activities of IFNs. The observation that IFNs can rapidly induce proteins that have the capacity to inhibit their actions implies that this could be an important negative feedback loop to limit the extent of IFN effects on a cell.

Past studies have demonstrated that *Socs1* and *Socs3* gene expression is inducible by a number of factors including IL6, LIF, GH and thrombopoietin (Endo *et al.*, 1997; Starr *et al.*, 1997; Naka *et al.*, 1997; Masuhara *et al.*, 1997; Adams *et al.*, 1998; Bousquet *et al.*, 1999; Ram & Waxman, 1999). The biological effects of these factors, similar to IFN effects can be negatively regulated by SOCS protein action (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997; Adams *et al.*, 1998). SOCS1 has been demonstrated *in vitro* to have the ability to bind to JAK1, 2, 3 and TYK2 via their central SH2 domains to the critical tyrosine within the JH1 domain of the JAKs (Endo *et al.*, 1997; Mashuhara *et al.*, 1997; Nicholson *et al.*, 1999; Yasukawa *et al.*, 1999). Binding to the JH1 domain of the JAKs, renders the JAKs unable to phosphorylate the receptors or latent transcription factors, resulting in the inhibition of signal transduction.

In vitro studies have shown SOCS1 and SOCS3 to be the most potent inhibitors of cytokine signaling (Song & Shuai, 1998; Sakamoto *et al.*, 1998). The *in vitro* studies represented in this chapter are of critical importance in readily demonstrating the dramatic and rapid induction of *Socs1* and *Socs3* gene expression in response to IFNs in particular type I. Both the magnitude and short period of induction of *Socs1* and *Socs3* gene expression by type I IFNs, IFNβ in particular is remarkable. Other ISGs such as *Mx1*, *Irf1* and others are often induced over longer time periods and do not portray the same magnitude of response in such a short time. These experiments have examined the mRNA expression of the *Socs* family members, which may not translate into

functional proteins in such a short time. Another explanation may be protein stability, if SOCS1 and 3 are relatively unstable or have a quick turnover rate, a high level of gene induction would be beneficial. However this data is somewhat conflicting with other studies that describe *Socs1* as a non-IFNβ inducible gene, and that *Socs3* gene expression is not induced by either IFNβ or IFNγ (Sakamoto *et al.*, 1998). The results presented by Sakamoto *et al.* (1998) demonstrated that at 12 and 24 hours post IFNβ treatment, neither *Socs1* nor *Socs3* gene expression was observed. However results presented in this chapter demonstrate that IFNβ dramatically induced the gene expression of *Socs1* and *Socs3*, which peaked 15 to 30 minutes after treatment then returned to basal levels by 4 hours and remained normal 12 and 24 hour after. Therefore different kinetics can account for the conflicting IFNβ results. The conflicting *Socs3* data are not due to kinetics however, may be due to cell specificity. Results presented here demonstrate a 9.5-fold increase 24 hours post IFNγ treatment in murine L929 cells compared to the total lack of expression presented by Sakamoto *et al.* (1998) in M1 cells.

The gene induction by IFNs of both *Socs1* and *Socs3* and subsequent protein synthesis provides a negative feedback loop in which the SOCS proteins may function. Overexpression studies presented have shown potential importance of the SOCS proteins, in particular SOCS1 and SOCS3, as important critical inhibitors of IFN signaling, in particular antiviral signaling which is crucial in host defence. The critical issue is whether these effects are relevant to the levels and kinetics of regulation of gene expression that occurs *in vivo*. Results presented imply that within 1 hour of IFN signaling, the pathway is capable of being switched off or dampened, which is usually before ISGs that are induced to act in an antiviral response (e.g. *Mx1*) have reached maximal induction levels. However, the kinetics of the SOCS proteins may be different from the transcripts, taking longer to translate or these proteins may have a high turnover rate due to a lack of long term stability. Studies have shown that the mechanisms involved in this negative regulation in the case of SOCS1 occurs at the level of the receptor complex, with SOCS1 binding *in vitro* to the JH1 domains of the activated JAKs to stop phosphorylation and any subsequent

activation, hence stopping any further signaling (Figure 3.8). An understanding of gene functions may be elucidated through *in vitro* studies, however understanding the *in vivo* role is of more importance, as the use of *in vivo* models can often highlight *in vitro* artifacts. From this chapter it has been elucidated *in vitro* that *Socs1* and *Socs3* are induced by and subsequently inhibit IFN antiviral responses, therefore given the importance of *in vivo* studies, the role of *Socs1* will be examined in a mouse model in the following chapters.

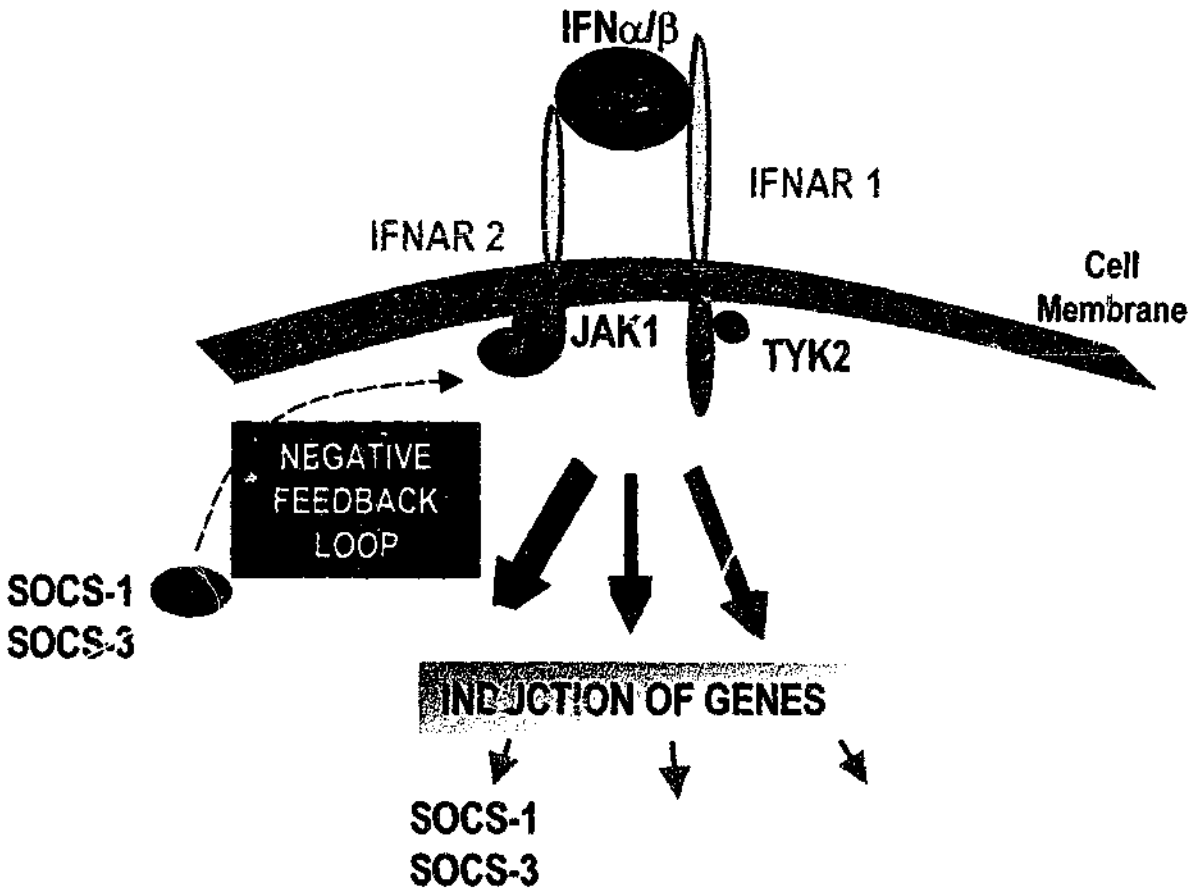


Figure 3.8: A Model for the Proposed Mechanism of *Socs1* and *Socs3* Function.

IFNs are able to induce the expression of the *Socs1* and *Socs3* genes which inhibit the antiviral actions of the same inducing IFNs.

CHAPTER 4

SOCS1 Regulation of Type I IFN Responses *in vivo*

4.1 INTRODUCTION

In order to gain an understanding of the *in vivo* role of SOCS1 in regulating type I IFN signaling a study was undertaken of genetically modified mice with a targeted mutation in the *Socs1* gene (Starr *et al.*, 1998). These mice have a very dramatic phenotype of neonatal lethality within 21 days of age. They die from a disease characterised by a fatty degeneration of the liver, monocytic and macrophage infiltration of several organs and multiple haematopoietic abnormalities including severe lymphopenia (Starr *et al.*, 1998; Metcalf *et al.*, 1999). The *Socs1*$^{-/-}$ mice have smaller thymi and lymphocytes that display an increased degree of apoptosis that is associated with high levels of BAX, a proapoptotic protein (Naka *et al.*, 1998). Therefore the generation of these mice deficient for *Socs1* have clearly demonstrated that *Socs1* is critical for postnatal development (Starr *et al.*, 1998).

Interestingly, all of the effects reported in the *Socs1*$^{-/-}$ mice, including neonatal death and hepatitis were induced in wild-type mice injected daily with IFNγ from birth (Gresser, 1982). The normal function of IFNγ is considered to be the activation of genes that function in antigen presentation, macrophage activation, antiproliferative and antiviral responses (Boehm *et al.*, 1997; Tatake & Zeff, 1993). However, excess systemic levels of IFNγ cause liver damage with decreased numbers of B cells, most likely due to increased levels of apoptosis (Gresser, 1982; Young *et al.*, 1997; Naka *et al.*, 1998). Studies examining IFNγ responses in the *Socs1*$^{-/-}$ neonates demonstrated higher expression levels of IFNγ induced ISGs in various tissues, and increased STAT1 activation in comparison to wild-type controls. This data suggested that the *Socs1*$^{-/-}$ mice were more sensitive to the effects of IFNγ (Alexander *et al.*, 1999). There were increased levels of IFNγ detected in the serum of the *Socs1*$^{-/-}$ mice but not in mice lacking *Socs1* and *Rag2* (required for active T cells) (Marine *et al.*, 1999). This implied that the lymphocytes from the *Socs1*$^{-/-}$ were responsible for the increased levels of IFNγ found in the serum. It was hypothesised that the phenotype of the *Socs1*$^{-/-}$ mice was due to excess IFNγ, resulting from deregulation of cytokine signaling which was further compounded by increased responses to IFNγ signaling (Starr *et al.*, 1998; Alexander *et al.*, 1999).

The crucial role of SOCS1 in balancing the lethal toxic effects of IFNγ with its beneficial immunological effects was validated when *Ifng*$^{-/-}$ and *Socs1*$^{+/-}$ mice were crossed to generate *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice. These mice were healthy and viable despite being deficient for *Socs1* and lacked any of the abnormalities initially reported with the fatal *Socs1*$^{-/-}$ phenotype (Alexander *et al.*, 1999). Rescuing the fatal phenotype of the *Socs1*$^{-/-}$ by deleting *Ifng* and hence removing all endogenous IFNγ demonstrated that IFNγ signaling was an important SOCS1 target. However, the wealth of *in vitro* data demonstrating that SOCS1 can negatively regulate signaling from other cytokines, imply that IFNγ may not be the exclusive SOCS1 target. Therefore, the generation of the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mouse model provided an *in vivo* model in which to study the effects of SOCS1 on other cytokines.

Since the type I IFNs were shown to be even more potent inducers of *Socs1* than IFNγ and SOCS1 to be a potent inhibitor of type I IFNs *in vitro*, a series of experiments were designed using the *Socs1*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice to determine whether SOCS1 was an important regulator of type I IFNs *in vivo*. As the signature action of type I IFNs is its rapid production and action in acute viral infections, we have chosen such a disease model as the focus of this study.

4.2 RESULTS

4.2.1 Genotyping of *Socs1* Gene Status

Due to the lethal phenotype of the *Socs1*$^{-/-}$ mice, all *in vivo* experiments were carried out using neonates from *Socs1* heterozygous matings. Genotyping was performed using genomic DNA extracted from the tails of neonatal mice aged 8-10 days and digested with *Eco*RI. The digested DNA was electrophoresed through 1% agarose gels and transferred to Genescreen Plus membranes (refer to 2.2.3.2). The membranes were hybridised with a ^{32}P-labelled 1.5 kb *Eco*RI/*Hind*III fragment of the *Socs1* gene (refer to 2.2.2). All three genotypes *Socs1*$^{+/+}$, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ have been observed from previous studies in a Mendelian 1:2:1 frequency (Starr *et al.*, 1998; Naka *et al.*, 1998). The wild-type *Socs1* allele was at the expected size of 5.3 kb, whilst the targeted allele was 8.0 kb (Figure 4.1).

4.2.2 *Socs1*$^{-/-}$ Neonates Display a Higher Resistance to *S*emliki *F*orest *V*irus (SFV) Infection.

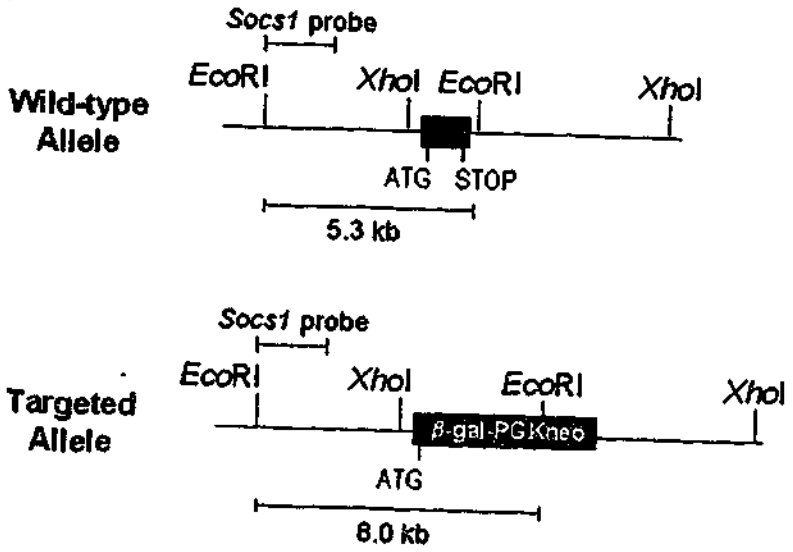
One of the many roles type I and II IFNs play is to co-ordinate a host response to viral infection. All experiments using the *Socs1* neonatal mice were conducted before they had been genotyped. Neonatal mice aged between 5-10 days were infected with SFV at 10 x TCID-50 and subsequently monitored with all fatalities being recorded in days post infection. Two days after infection 15% of wild-type (2/13) and 20% of *Socs1*$^{+/-}$ (5/25) mice were resistant, then by 3 days no wild-type (0/13) or *Socs1*$^{+/-}$ (0/25) mice were resistant to SFV infection (Figure 4.2). In contrast, 100% of neonates lacking the *Socs1* gene (10/10) were resistant 2 days after infection, by 10 days 50% (5/10) were still exhibiting resistance. By 21 days post infection 0% (0/10) mice were able to survive, which was statistically different from the resistance exhibited by wild-type and *Socs1*$^{+/-}$ neonates as determined by a Logrank test (P<0.001). This result suggested a complete resistance to SFV infection in the *Socs1*$^{-/-}$ mice was not

Figure 4.1: Genotyping of Neonatal Mice with a Targeted Mutation of the *Socs1* Gene.

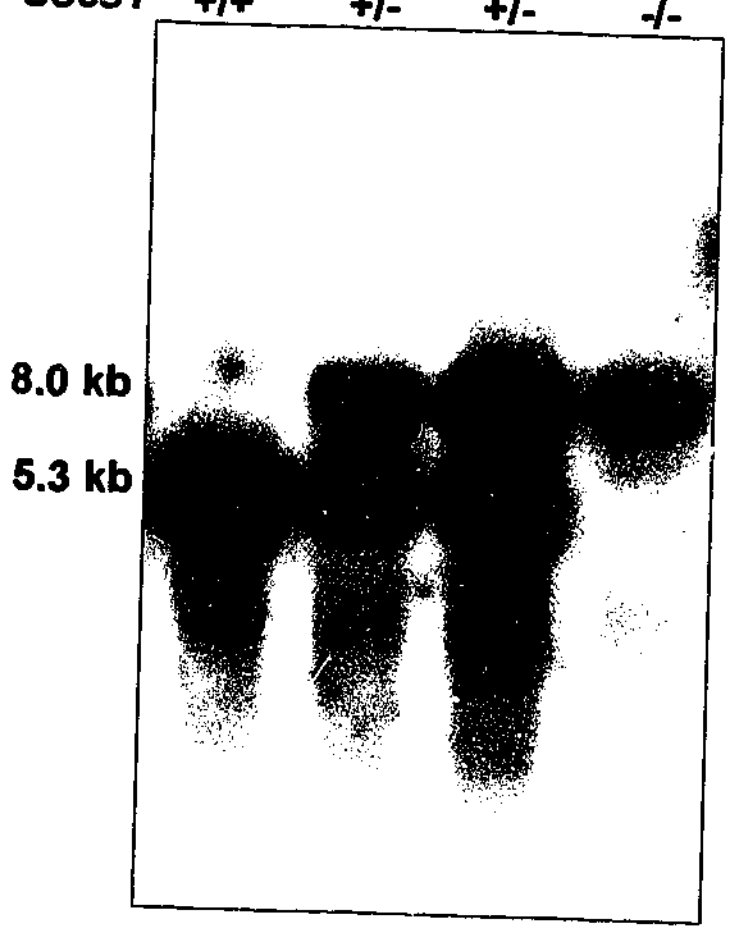
A. A schematic representation of the targeting strategy of the *Socs1* allele. The wild-type *Socs1* allele contains a single exon which was replaced by a β-gal-PGK neo cassette which was fused to the ATG of the *Socs1* gene. The probe used to detect the wild-type and targeted alleles was 5' of the ATG (Starr *et al.*, 1998).

B. Southern blot of *Eco*RI digested genomic DNA from $Socs1^{+/+}$, $Socs1^{+/-}$ and $Socs1^{-/-}$ neonates produced from $Socs1^{+/-}$ matings. Filters were hybridised with a 1.5 kb *Eco*RI/*Hind*III fragment as described in 2.2.2. The expected band sizes were 5.3 kb and 8.0 kb for the wild-type and $Socs1^{-/-}$ alleles respectively.

A.


Socs1 probe
Wild-type Allele
EcoRI
XhoI
EcoRI
XhoI
ATG
STOP
5.3 kb
Socs1 probe
Targeted Allele
EcoRI
XhoI
EcoRI
XhoI
β-gal-PGKneo
ATG
8.0 kb

B.


Socs1
+/+
+/-
+/-
-/-
8.0 kb
5.3 kb

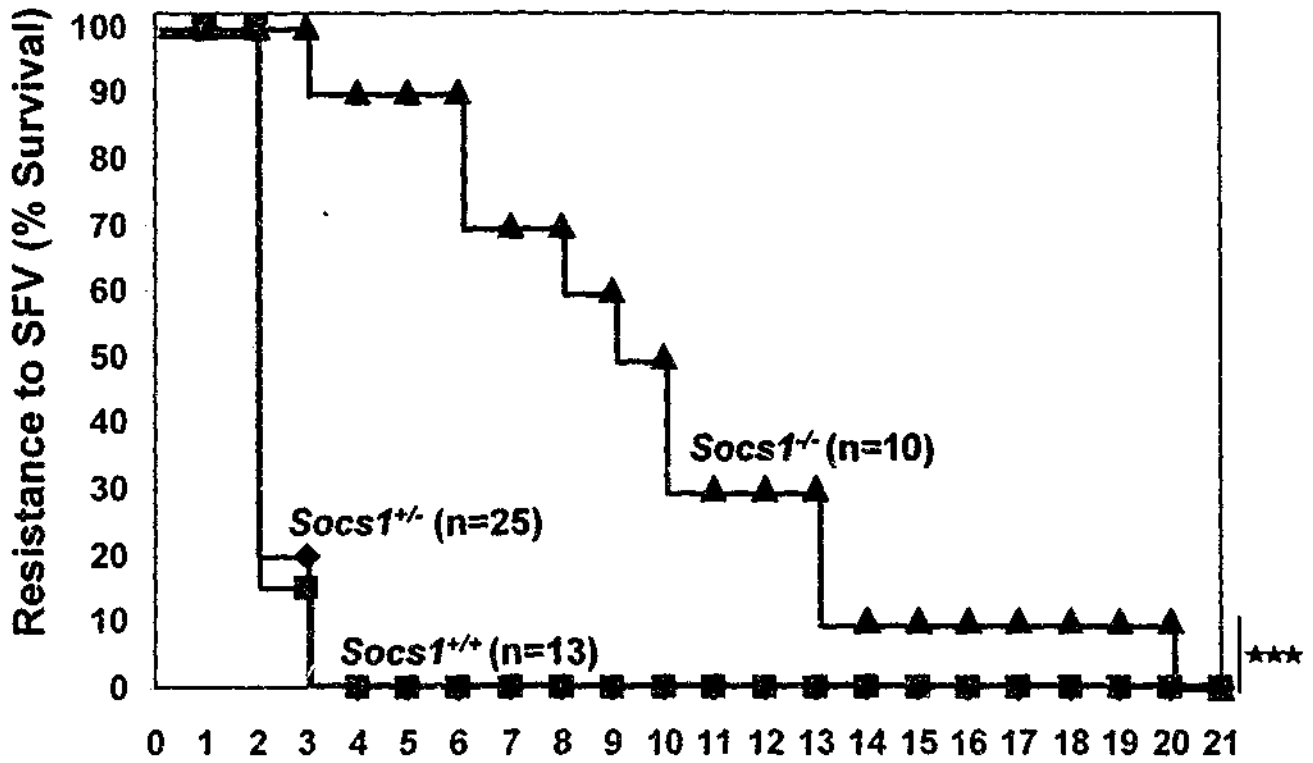


Figure 4.2: Neonatal Mice with Targeted Mutation of the *Socs1* Gene Display a Higher Resistance to SFV Infection.

Survival plot measuring the resistance of SFV infected neonates aged between 5-10 days at 10 x TCID-50. Neonates were monitored with all fatalities recorded. *Socs1*$^{+/+}$ —■— (n=13) or *Socs1*$^{+/-}$ —◆— (n=25) neonates succumbed to infection within 3 days. In contrast, *Socs1*$^{-/-}$ —▲— (n=10) neonates were significantly more resistance to SFV infection surviving for as long as 21 days (***P<0.001) as determined by a Logrank test.

significantly different to the survival of the *Socs1*$^{-/-}$ mice in response to SFV infection.

4.2.3 Viral Titres in the *Socs1*$^{-/-}$ Neonates are Significantly Lower than their Wildtype and Heterozygous Littermates

There are numerous possible reasons for the increased resistance to SFV observed in the *Socs1*$^{-/-}$ neonates. There may be increased amounts of IFN being produced. There may also be differences in the kinetics of the antiviral response, either in amplitude or length of response, all of which will be considered. IFNs are known to interfere with viral replication (Issac & Lindemann, 1957; Stites *et al.*, 1997), therefore measuring viral replication will ascertain the involvement of IFNs over any other pro-survival mechanism. *Socs1*$^{-/-}$ neonates, generated from *Socs1* heterozygous matings aged between 5 and 10 days were used to measure the amount of viral replication occurring after acute infection with SFV. This was achieved by injecting SFV at 10 x TCID-50 into the intraperitoneal cavity and subsequently monitoring the health of the mice over the proceeding 48 hours. The TCID-50 was defined by a CPE assay as the dose that was fatal to 50% of L929 cells in culture. Previous titration *in vivo* (Hwang *et al.*, 1995) determined that a 10 x TCID-50 dose would be appropriate in order to complete experiments on *Socs1*$^{-/-}$ neonatal mice in a short time period before they started to die from genotype related causes. After 48 hours, the neonates were killed and the spleen, kidneys, liver, thymus and lungs were harvested for testing viral titres. Blood samples were taken from the neonates and serum was collected for testing IFN and virus levels.

The organs were weighed and then homogenised for use in CPE assays to measure the viral titres (refer to 2.5.6.1). The growth patterns of the *Socs1*$^{-/-}$ neonates did not differ from their wild-type or heterozygous littermates until after 10 days of age, after which time their growth tends to become stunted along with overall reduction in organ size (Starr *et al.*, 1998). Therefore the lack of

significant differences in the weights of each of the organs between the *Socs1*$^{-/-}$ and the *Socs1*$^{+/+}$ and *Socs1*$^{+/-}$ littermates was consistent with published data (Figure 4.3A).

Virus was detected in all organs from wild-type, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ neonates. There were no differences observed between the viral titres from any of the organs tested from heterozygous or wild-type neonates. However, in all cases the viral titres in the *Socs1*$^{-/-}$ neonates were significantly lower than that of the wild-type and heterozygous littermates (Figure 4.3B). Wild-type lungs had up to 1000-fold higher viral titres with a mean +/- SEM of 6.2+/-0.36 compared to 3.0+/-0.59 in the *Socs1*$^{-/-}$ (P<0.001). The spleen from wild-types had up to 100-fold higher viral titres of 3.6+/-0.29 compared to 1.3+/-0.30 in *Socs1*$^{-/-}$ neonates (P<0.001). The kidneys from wild-types had up to 100-fold higher viral titres of 4.59+/-0.26 compared to 2.38+/-0.59 in *Socs1*$^{-/-}$ kidneys (P<0.01). Wild-type serum had up to 1000-fold higher viral titres of 4.17+/-0.43 compared to 1.41+/-0.52 in *Socs1*$^{-/-}$ serum (P<0.01). The thymus from wild-type neonates had up to 100-fold higher viral titres of 4.67+/-0.31 compared to 1.92+/-0.62 in *Socs1*$^{-/-}$ neonates (P<0.01). Finally the liver demonstrated up to 10-fold higher viral titres of 6.01+/-0.28 in the wild-types compared with 4.22+/-0.72 in the *Socs1*$^{-/-}$ littermates (P<0.05). These results demonstrate that 48 hours post infection with SFV, that the *Socs1*$^{-/-}$ neonates are able to reduce the viral titres in their organs, which is consistent with the amplified antiviral response which could be due to an increased IFN signal. As the viral replication has decreased it suggests that IFNs are responsible, however this increased signal could be due to either increased IFN production and/or an increased sensitivity to IFN.

4.2.4 IFN Levels in Serum from SFV Infected Neonates

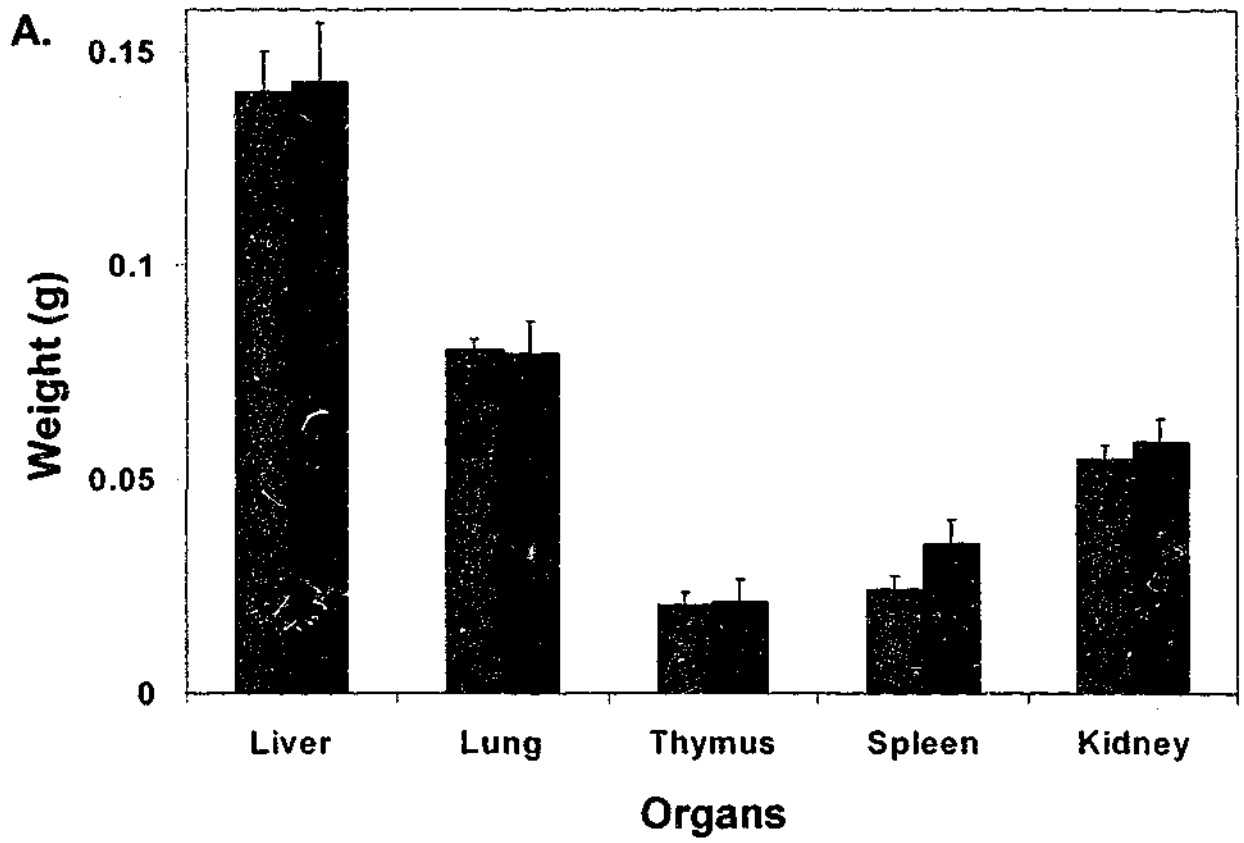
Since type I IFN is known to be induced after viral infection, we examined whether the lack of *Socs1* caused an increase in type I IFN levels. In untreated healthy mice, serum levels of IFN are generally undetectable. However, following viral challenge, expression of type I IFNs are rapidly induced, resulting

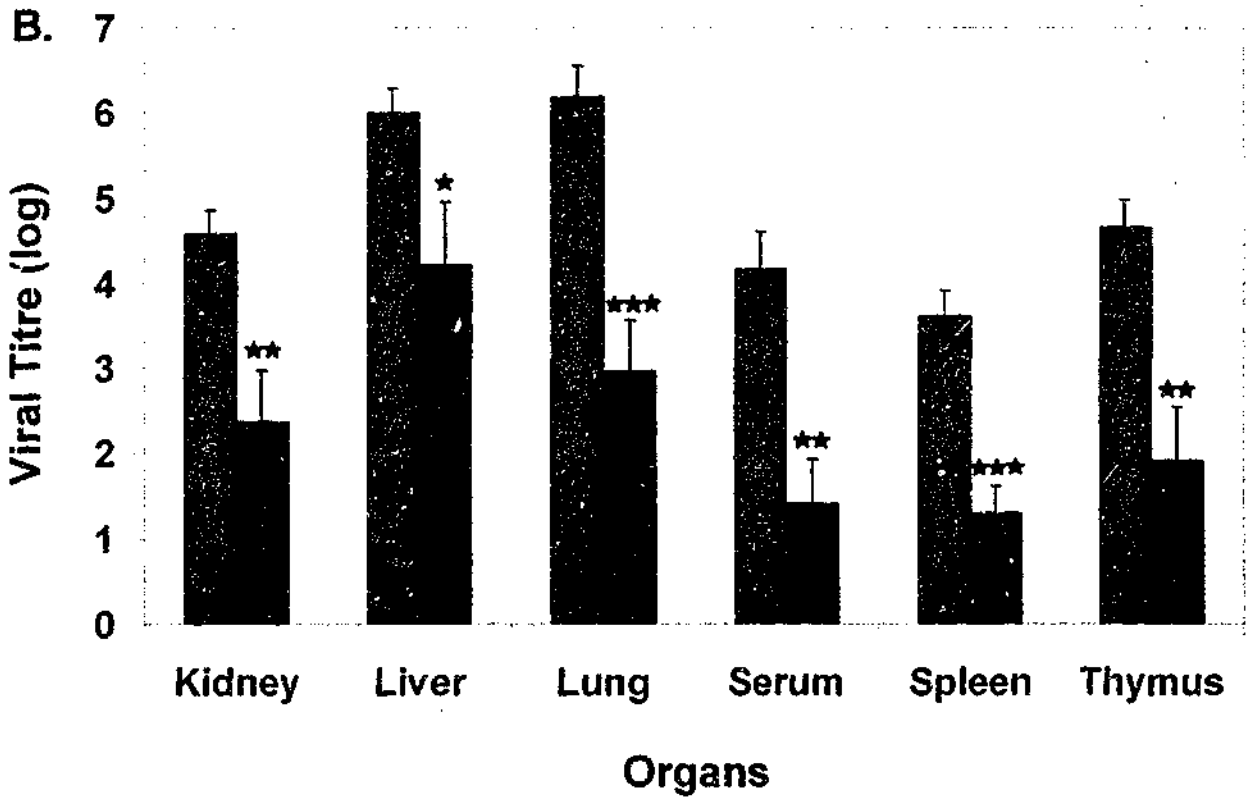
Figure 4.3A: Organ Weights of SFV Infected Neonatal Mice with Targeted Mutation of the *Socs1* Gene.

Socs1$^{-/-}$ (n=9) and wild-type/heterozygous neonate littermates (n=40) between 5-10 days of age were infected with 10 x TCID-50 SFV. Neonates were monitored for 48 hours at which time they were killed and the liver, lungs, thymus, spleen, kidneys and brain harvested and weighed. There was no significant difference in the weights of these organs between wild-type ■ and *Socs1*$^{-/-}$ neonates ■.

Figure 4.3B: Viral Replication in Neonatal Mice with Targeted Mutation of the *Socs1* Gene.

Neonates produced from *Socs1*$^{+/-}$ matings were injected with SFV at 10 x TCID-50 when they were between 5-10 days of age. Neonates were observed for 48 hours at which time they were killed and organs harvested to measure viral loads. Viral titres were measured by CPE assays as described in 2.5.6.1, and expressed as mean log titres +/- SEM. *Socs1*$^{-/-}$ neonates ■ (n=9) had significantly lower viral titres in the mentioned organs when compared to *Socs1*$^{+/+}$ and *Socs1*$^{+/-}$ littermates ■ (n=40) (⋆P<0.05, ⋆⋆P<0.01, ⋆⋆⋆P<0.001).


A.
0.15
0.1
0.05
0
Weight (g)
Liver
Lung
Thymus
Spleen
Kidney
Organs


B.
7
6
5
4
3
2
1
0
Viral Titre (log)
**
*
***
**
***
**
Kidney
Liver
Lung
Serum
Spleen
Thymus
Organs

in readily detectable levels of these cytokines in serum (Hertzog *et al.*, 1988). Therefore the levels of IFN (combined type I and II) were measured in serum samples from SFV infected wild-type and *Socs1*$^{-/-}$ neonates using a bioassay with murine L929 cells as targets for SFV challenge (refer to 2.5.6.2). Forty-eight hours after SFV intraperotineal infection 24 out of 31 (77%) wild-type and *Socs1*$^{+/-}$ neonates had elevated levels with a mean of 30 units/ml ranging up to approximately 1000 units/ml of IFN in their serum. In contrast, IFN was only detected in the serum of 3 out of 9 (33%) samples with a mean of 3 units/ml ranging up to 300 units/ml in *Socs1*$^{-/-}$ neonates 48 hours post infection. Therefore the *Socs1*$^{-/-}$ neonates generally had lower levels of IFN in their serum (Figure 4.4). Statistical analysis using Mann-Whitney rank sum tests indicated that the difference observed was significant ($P<0.05$). The levels of IFN detected would most likely be type I IFN levels as IFNγ is induced indirectly by cytokine mechanisms rather than by virus itself as the type I IFNs are (Sen, 2001). The levels of IFN measured in these mice after infection are higher than the levels of IFNγ in unstimulated mice (Marine *et al.*, 1999). This result implies that the increased resistance observed in the *Socs1*$^{-/-}$ neonates to SFV infection is not caused by an increase in the production of IFN. Therefore it is likely that the higher resistance is due to an increase in sensitivity to antiviral actions of IFNs. However as both type I and II IFNs have antiviral capabilities, either IFNα/β or IFNγ may be responsible.

4.2.5 *Ifng*$^{-/-}$ Neonates are as Sensitive to Infection with SFV as Wildtype Littermate Controls

IFNγ is an important pro-inflammatory cytokine that contributes to antiviral immunity (Dalton *et al.*, 1993; Bohem *et al.*, 1997; Stark *et al.*, 1998). Because SOCS1 has previously been shown to regulate responses to IFNγ (Alexander *et al.*, 1999; Brysha *et al.*, 2001), the question of whether the increased antiviral response in the *Socs1*$^{-/-}$ neonates could be attributed to an increase in IFNγ sensitivity addressed. Firstly, in order for this to be examined, *Ifng*$^{-/-}$ neonates along with wild-type neonates were infected with SFV at 10 x TCID-50 and

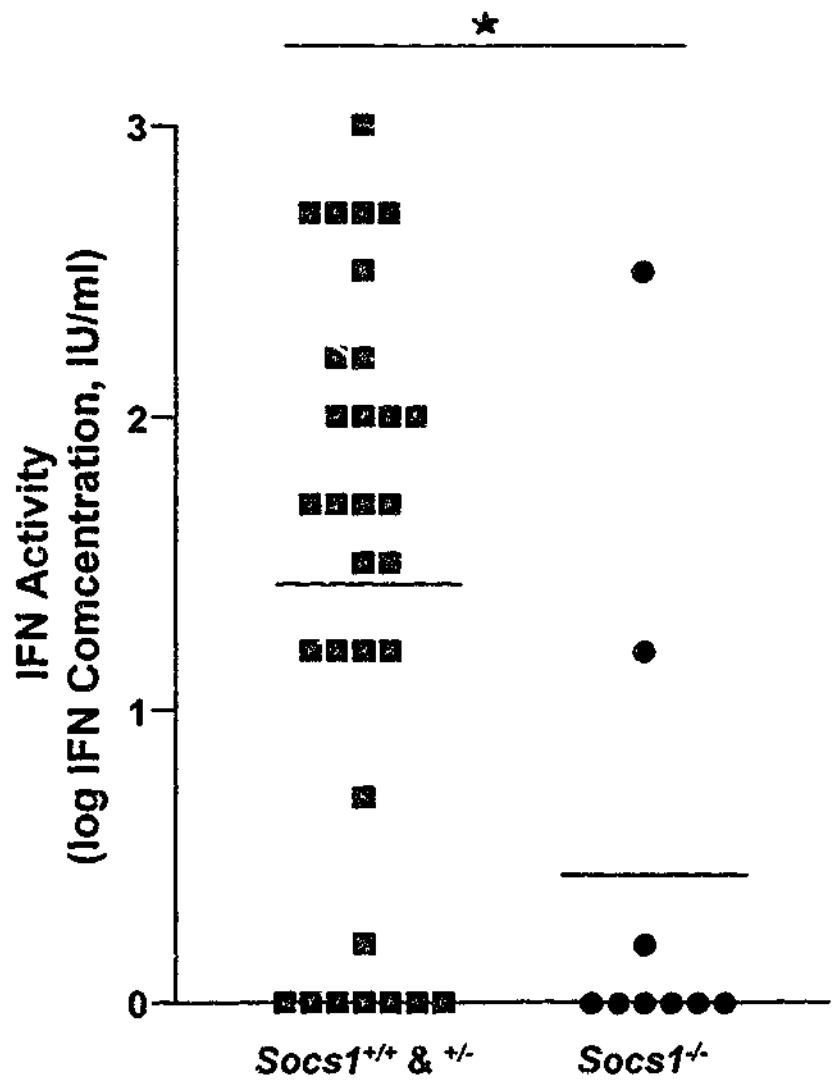


Figure 4.4: IFN Levels in Serum from SFV Infected *Socs1*$^{+/+}$, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ Neonates.

Neonates produced from *Socs1*$^{+/-}$ matings were injected with SFV at 10 x TCID-50 between 5-10 days of age. Neonates were observed for 48 hours at which time blood was collected and serum extracted to measure IFN levels using a CPE reduction assay as described in 2.5.6.2. Each point represents individual mice with the mean levels represented by the lines. *Socs1*$^{-/-}$ neonates ● (n=9) had significantly lower serum levels of IFN compared to their *Socs1*$^{+/+}$ and *Socs1*$^{+/-}$ littermates ■ (n=31) determined by a Mann-Whitney rank sum test (★ $P<0.05$).

resistance measured as % survival post infection. There was no observable difference measured by Logrank tests between the sensitivity of wild-type neonates with 13/13 dying within 4.25 days compared to that of 16/16 *Ifng*$^{-/-}$ neonates dying within 4 days post infection (Figure 4.5). This result demonstrated that this virus was not sensitive to IFNγ, implying that the amplified antiviral response seen in the *Socs1*$^{-/-}$ neonates to SFV was independent of the antiviral actions of IFNγ.

4.2.6 *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ Neonates are more Resistant to SFV Challenge than Wild-type Neonates

By utilising the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ double knockout mice it can be determined conclusively if the increased resistance demonstrated by the *Socs1*$^{-/-}$ neonates is in fact due to a hypersensitivity to IFNγ or IFNα/β. When the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates were infected with SFV at 10 x TCID-50, they were resistant as 5 out of the 6 neonates infected (84%) survived past 22 days. In contrast, 0 out of the 4 wildtypes infected (0%) survived, all dying within 7 days of infection (Figure 4.6A). Even when the concentration of virus was increased to 30 x TCID-50, 4 out of 8 (50%) of *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates survived 10 days post infection again compared to 0 out of 4 (0%) wild-type controls, all of which died within 4 days of infection (Figure 4.6B). In both mentioned experiments, the differences observed in the resistance to both 10 x or 30 x TCID-50 between the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and wild-type neonates was significantly different as determined by Logrank tests (P<0.01 and P<0.001 respectively).

This experiment has measured the resistance of the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates to SFV viral infection and has demonstrated again that IFNγ is not impacting on the response as the same response is observed in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ neonates. The type I IFNs are known to elicit responses in cases of acute viral infection, therefore by utilising the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates we can further elucidate the role of SOCS1 in regulating these type I IFN responses to viral infection

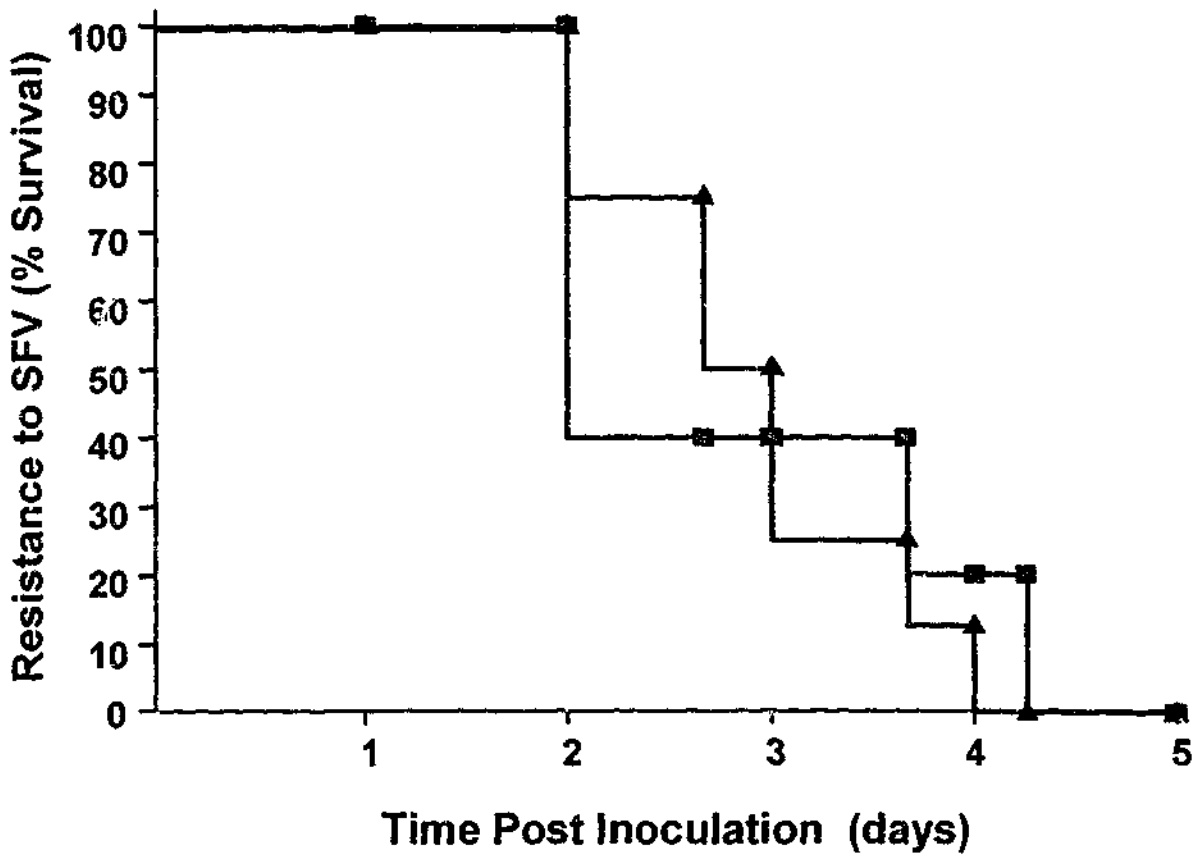


Figure 4.5: Semliki Forest Virus (SFV) Infection in *Ifng*$^{-/-}$ Neonates.

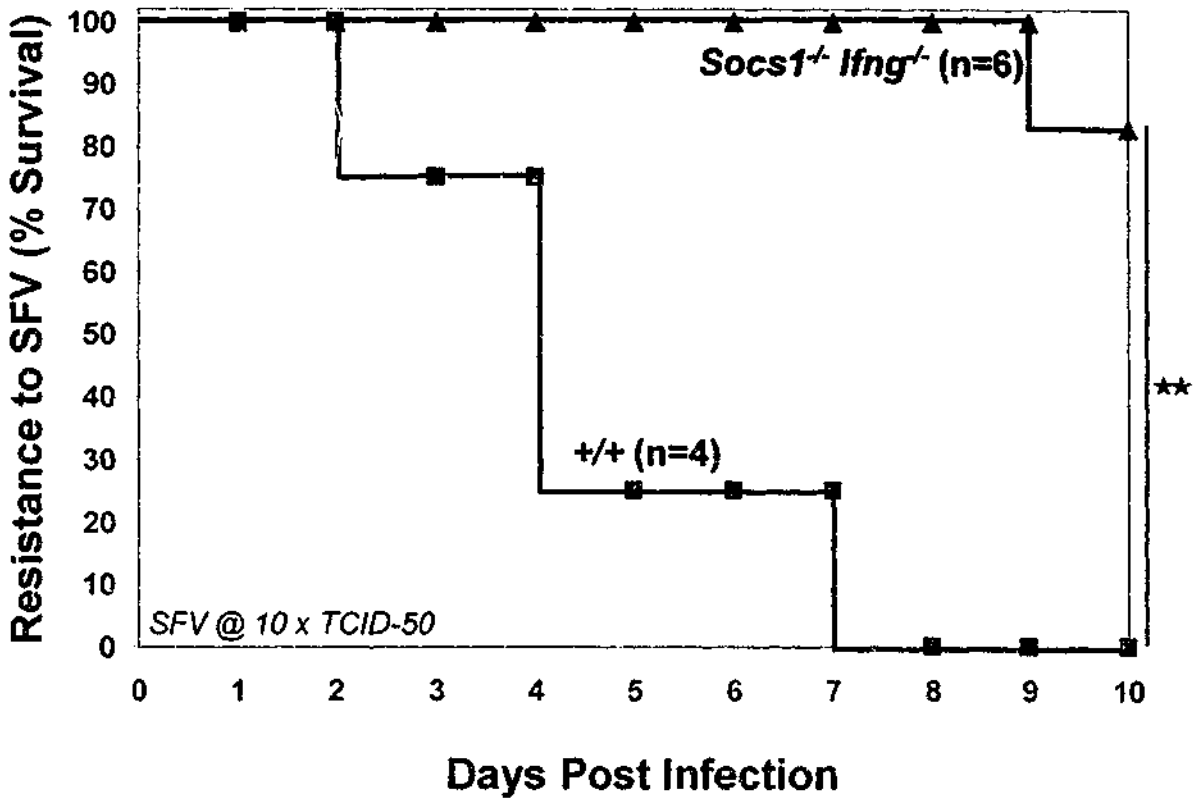
Survival plot measuring the resistance of SFV infected wild-type -■- (n=13) and *Ifng*$^{-/-}$ -▲- (n=16) neonates aged between 4-11 days at 10 x TCID-50. Neonates were monitored with all fatalities recorded. There was no statistical difference observed in resistance to SFV between wild-type and *Ifng*$^{-/-}$ neonates as measured by a statistical Logrank test.

Figure 4.6: *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ Neonates Display a Higher Resistance to SFV than Wild-type Controls.

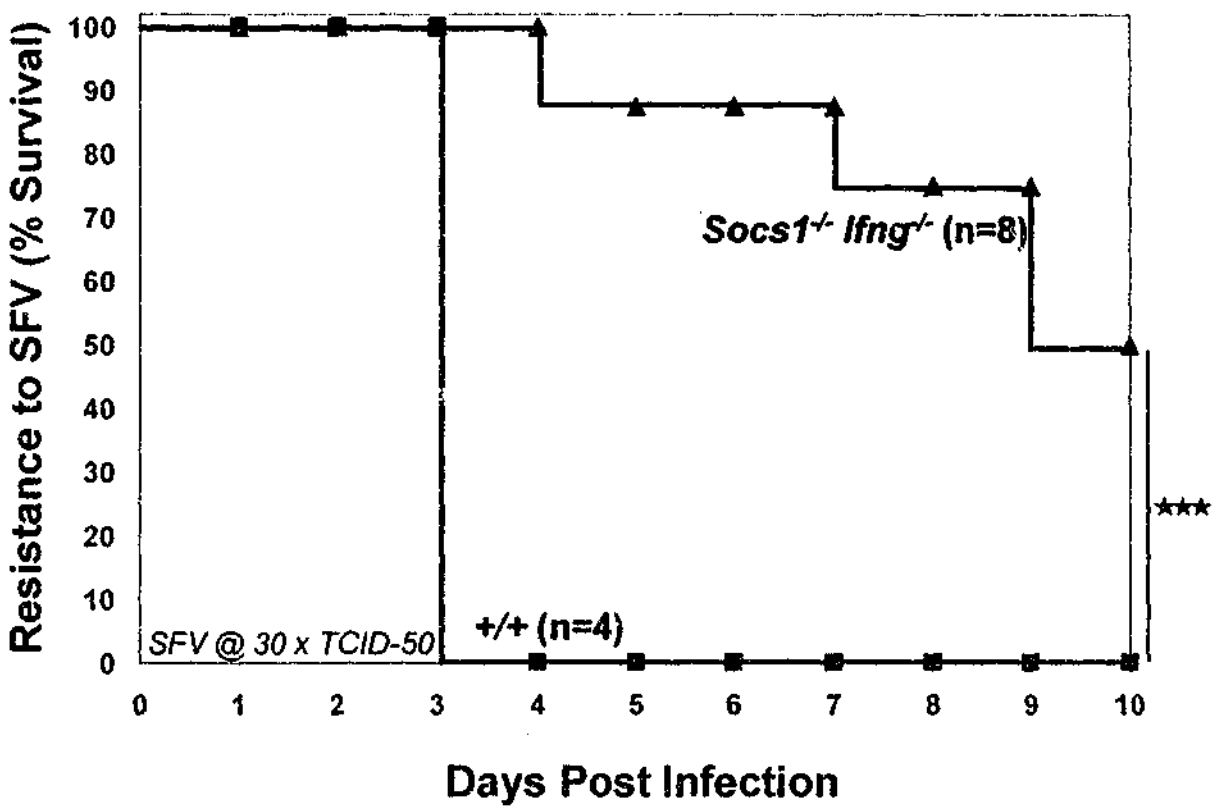
A. Survival plot measuring resistance of SFV infected wild-type —■— (n=4) and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ —▲—(n=6) neonates aged between 5-10 days at 10 x TCID-50. The health of the neonates was monitored and fatalities recorded. Wild-type neonates died within 7 days while 5/6 double knockout neonates survived 22 days post infection which was statistically significant as measured by a Logrank test (★★P<0.01).

B. Survival plot measuring the resistance of SFV infected wild-type —■—(n=4) and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ —▲—(n=8) neonates aged between 5-10 days at 30 x TCID-50. The health of the neonates was monitored and fatalities recorded. Wild-type neonates died within 4 days while 4/8 double knockout neonates survived 10 days post infection which was statistically significant as measured by a Logrank test (★★★P<0.001).

A.


Resistance to SFV (% Survival)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Days Post Infection
Socs1-/- Ifng-/- (n=6)
+/+ (n=4)
SFV @ 10 x TCID-50
**

B.


Resistance to SFV (% Survival)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Days Post Infection
Socs1-/- Ifng-/- (n=8)
+/+ (n=4)
SFV @ 30 x TCID-50
***

4.2.7 *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ Neonates have Lower Viral Titres than Wild-type Neonates

When the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates were infected with SFV they appeared to mimic their natural survival with the majority of neonates having the ability to survive what are lethal doses of virus to wild-type littermate controls (Alexander *et al.*, 1999; Figure 4.6). This phenomenon was also observed in *Socs1*$^{-/-}$ neonates, which display similar viral resistance and natural survival curves (Figure 4.2; Starr *et al.*, 1998). In order to demonstrate a direct effect on viral replication typical of IFNs, the antiviral response to SFV infection was ascertained by measuring viral titres in various organs of *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates. Neonates of both wild-type and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ genotypes aged between 5 and 10 days were infected with SFV intraperitoneally with 30 x TCID-50. After 48 hours all mice were killed and the liver, lungs, thymus, spleen, kidneys, brain and serum from blood were harvested and weights of all organs recorded. Again there were no significant differences observed in the weights of any of the organs from the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates compared to age matched wild-type controls (Figure 4.7A).

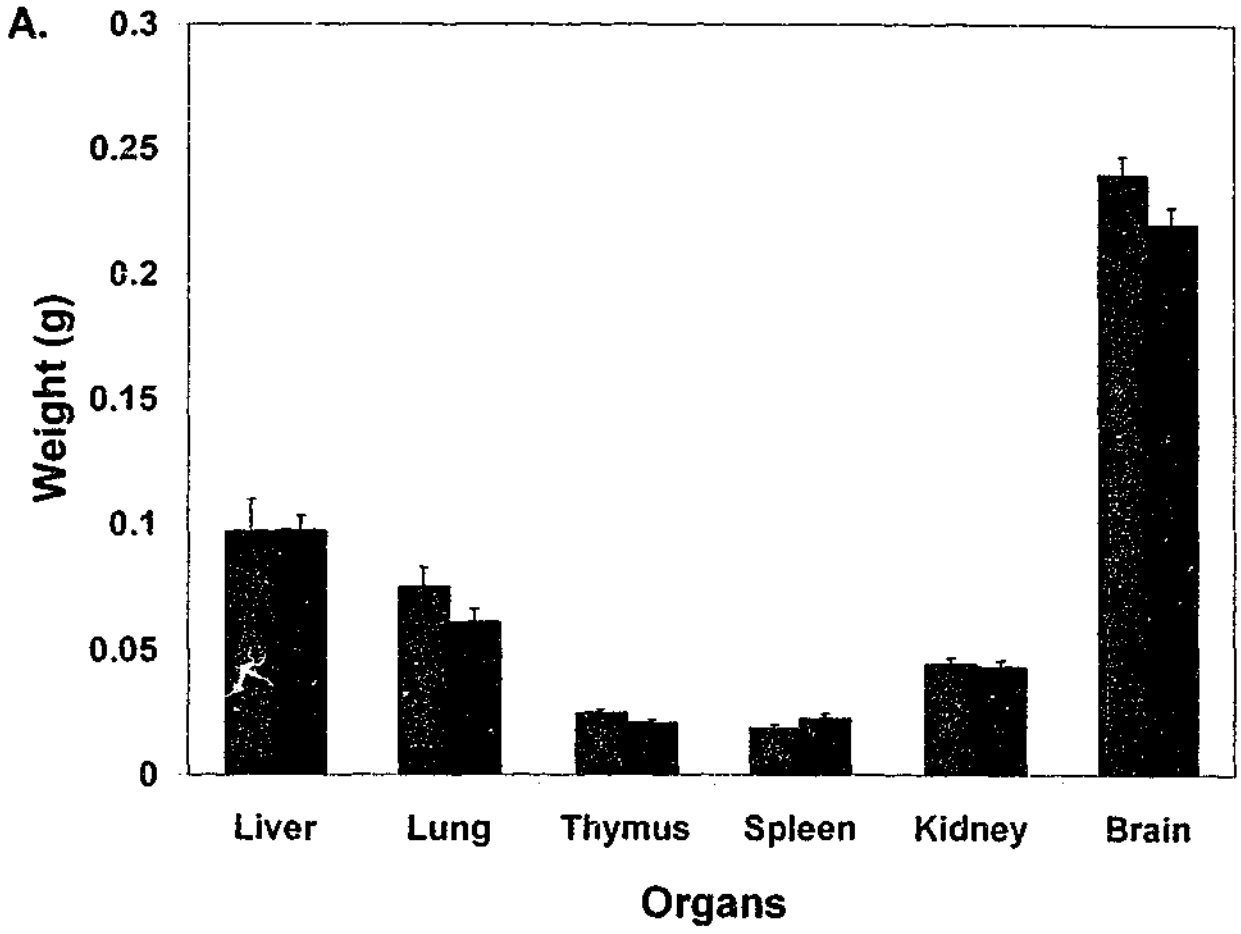
As before, the viral titres were measured using CPE assays. The levels of virus were not uniform across the organs tested as seen in the viral replication studies carried out on the *Socs1*$^{-/-}$, *Socs1*$^{+/+}$ and $^{+/-}$. However in all cases the viral titres in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates were lower than in the wild-type controls (Figure 4.7B). The viral titres measured in the wild-type lung were up to 100-fold higher with a mean +/- SEM of 2.48+/-0.95 compared to 0.65+/-0.40 in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$. The wild-type spleen had up to 100-fold higher viral titre of 1.85+/-0.65 compared to 0.13+/-0.04 in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$. Statistical analysis using Mann-Whitney rank sum tests indicated that the difference seen was significant (P<0.01). The kidneys displayed up to 100-fold higher viral titre of 0.65+/-0.45 in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ compared with titre of 2.53+/-1.16 in age matched wild-type controls. Again the Mann-Whitney rank sum test was used to demonstrate this difference was significant (P<0.05). The remaining organs all displayed lower viral titres in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ than in the wild-type controls,

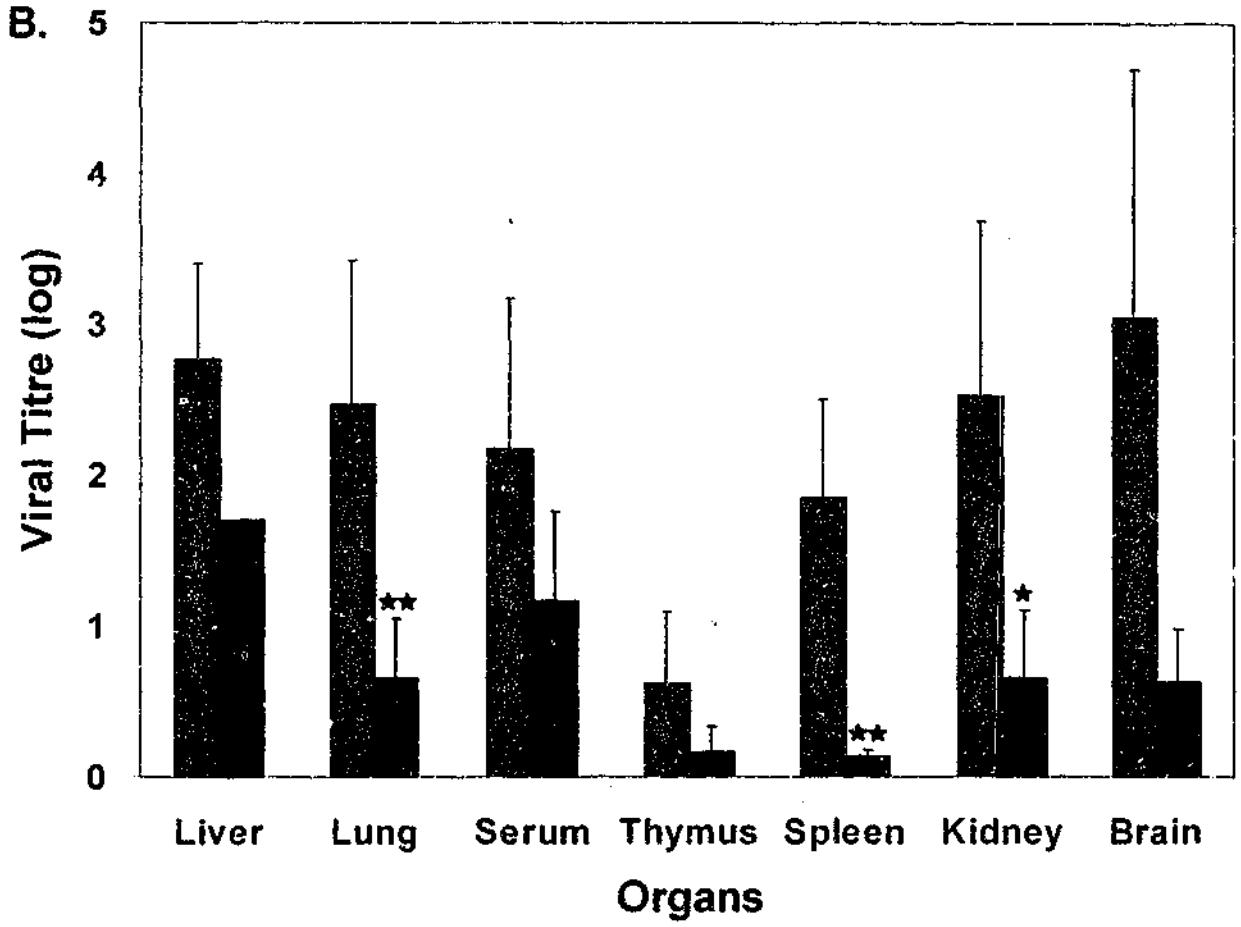
Figure 4.7A: Organ Weights of SFV Infected Neonatal Mice with Targeted Mutations of the *Socs1* and *Ifng* Genes.

Socs1$^{-/-}$ *Ifng*$^{-/-}$ ■ (n=6) and age-matched wild-type neonates ■ (n=4) between 5-10 days of age were infected with 10 x TCID-50 SFV. Neonates were monitored for 48 hours at which time they were killed and the liver, lungs, thymus, spleen, kidneys and brain harvested weighed. There was no significant difference in the weights of these organs between wild-types and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates.

Figure 4.7B: Viral Replication in Neonatal Mice with Targeted Mutations of the *Socs1* and *Ifng* Genes.

Socs1$^{-/-}$ *Ifng*$^{-/-}$ neonates were injected with the SFV at 30 x TCID-50 when they were between 5-10 days of age. Neonates were observed for 48 hours at which time they were killed and organs harvested to measure viral titres. Viral titres were measured by CPE assays as described in 2.5.6.1, and expressed as mean log titres +/- SEM. *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates ■ (n=6) had significantly lower viral titres as determined by Mann-Whitney rank sum tests in the kidneys, lungs and the spleen when compared to age matched wild-type controls ■ (n=4) (* $P<0.05$, ** $P<0.01$). In the remaining organs examined the trend of lower viral titres was exhibited in the double knockouts.


A.
0.3
0.25
0.2
0.15
0.1
0.05
0
Weight (g)
Liver
Lung
Thymus
Spleen
Kidney
Brain
Organs


B.
5
4
3
2
1
0
Viral Titre (log)
**
**
*
Liver
Lung
Serum
Thymus
Spleen
Kidney
Brain
Organs

however this did not reach statistically significant levels as a result of a wide range of control values. Therefore from this set of experiments it could be concluded that in the presence of acute viral infection, mice that lack both *Socs1* and *Ifng* had lower viral titres implying viral replication was less than in wild-type controls.

4.2.8 Type I IFN Mediates Resistance to SFV in *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ Neonates

The results presented thus far suggest that the type I IFNs are responsible for the heightened antiviral response observed in both the *Socs1*$^{-/-}$ and the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates by virtue of their lower viral titres after acute viral infection. To prove conclusively that the type I IFNs were responsible, 1000 IU of sheep anti-IFNα/β neutralising antibodies were injected into the intraperitoneal cavity of 5-10 day old *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates 6 hours prior to SFV infection (100 x TCID-50). As controls *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates of the same age were injected with the same volume of PBS 6 hours prior to SFV infection. All mice were subsequently monitored for fatalities. Wild-type neonates of the same age were also infected with the same virus and monitored as a positive control. Three days post infection 100% (5/5) of these wild-type neonates survived, by 4 days all of the wild-type neonates had died. In the absence of antibody treatment, 100% (11/11) of the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates were still alive 3 days after infection with 55% (6/11) of them surviving 10 days post viral challenge. In comparison pretreatment of the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates with the neutralising anti-IFNα/β antibody reversed the resistance of these mice to SFV infection with 22% (2/9) of *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates surviving 3 days after infection, however by 3.5 days 100% (9/9) of these neonates died (Figure 4.8). The differences observed in the resistance to SFV infection in the presence of neutralising IFNα/β antibodies were significantly different to the resistance observed in the absence of IFNα/β antibodies as determined by a Logrank test (P<0.001).

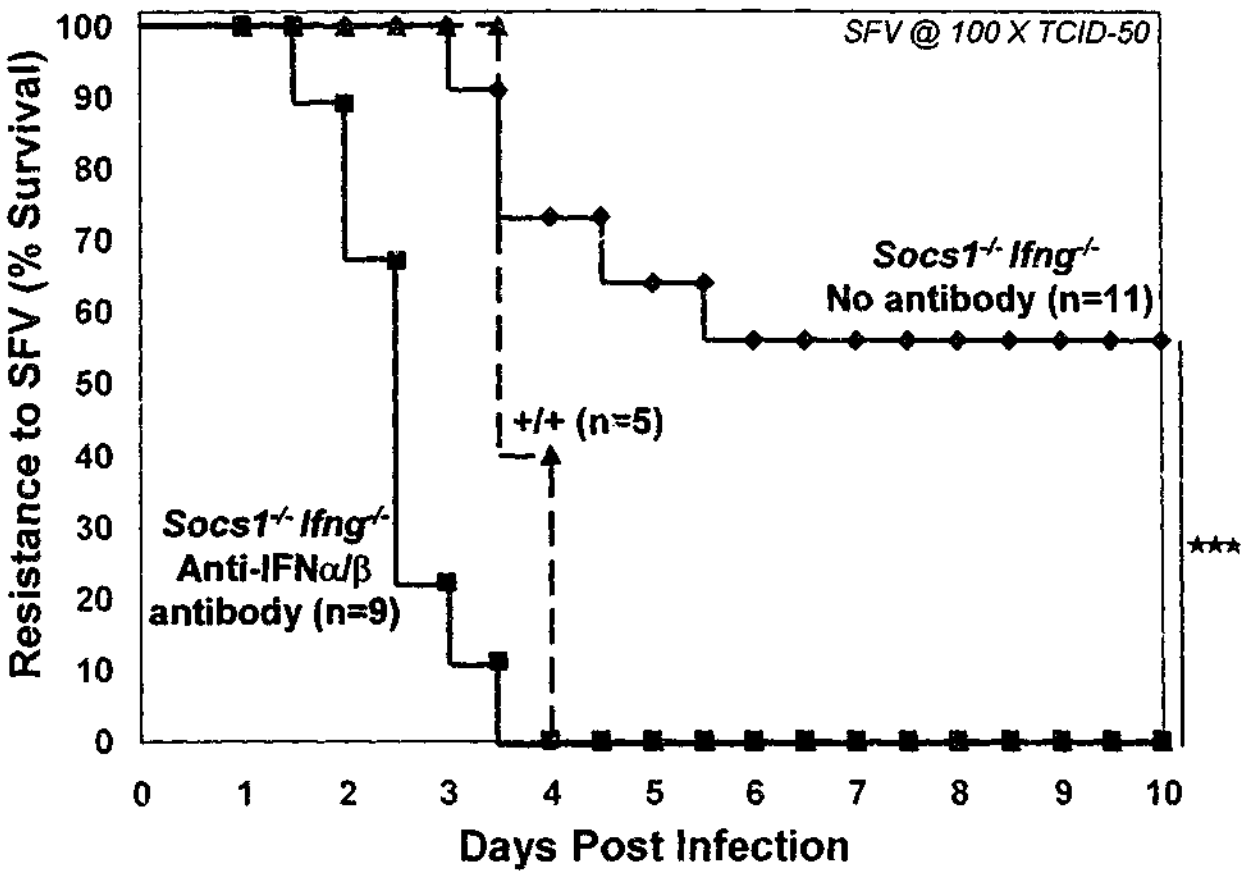


Figure 4.8: Survival after SFV Infections in *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ Neonates is due to the Type I IFN Antiviral Response.

Socs1$^{-/-}$ *Ifng*$^{-/-}$ neonates aged between 5-10 days were divided into two groups: control –◆– (n=11) and test –■– (n=9). The test group was administered 1000 IU sheep anti mouse IFNα/β antibody while the control group was injected with PBS. After 6 hours, both groups were infected with SFV at 100 x TCID-50 and monitored. Wild-type neonates –▲– (n=5) of the same age were infected with SFV at 100 x TCID-50 and monitored as a positive control. The administration of anti mouse IFNα/β antibody to *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice diminished resistance to viral infection significantly in comparison to no antibody as determined by Logrank tests (***P<0.001).

Therefore by blocking type I IFNs using of anti-IFNα/β antibodies, it could be concluded that the amplified antiviral response observed in both the *Socs1*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates was due to the effects of type I IFNs and not type II IFN. Interestingly, the wild-type controls were significantly more resistant to SFV infection than the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates administered the neutralising IFN antibody as measured by Logrank tests (P<0.05). This is consistent with the idea that the type I IFNs are important in acute viral infections regulating the response in wild-types even though they die.

4.2.9 Deletion of *Socs1* from *Ifnar1*$^{-/-}$ Neonates Increases their Resistance to Viral Infection

The *Ifnar1*$^{-/-}$ mice have been reported to be highly susceptible to viral infection (Müller *et al.*, 1994; Hwang *et al.*, 1995). By neutralising type I IFNs with an antibody, resistance to viral infection was reduced. Therefore by using a mouse that lacked one component of the type I IFN receptor would demonstrate again the importance of type I IFNs in acute viral infection, as these mice have reduced type I IFN signaling capabilities (Hwang *et al.*, 1995). *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$, *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ and *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were infected with SFV and resistance measured in the following days post infection. Results demonstrated that 100% (13/13) *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ and *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ neonates died within 24 hours post infection which was significantly different to the 33% (1/3) of *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice that died 2.5 days post infection. However, by 5.5 days post infection, the remaining 66% (2/3) of *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice died (P<0.001) (Figure 4.9). This suggested that *Socs1* regulated antiviral responses that signaled via IFNAR2, as the *Ifnar1*$^{-/-}$ mice were able to sustain a prolonged resistance to SFV infection. It also adds to the conclusion that the increased resistance observed in both the *Socs1*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates is in fact due to the antiviral actions of the type I IFNs.

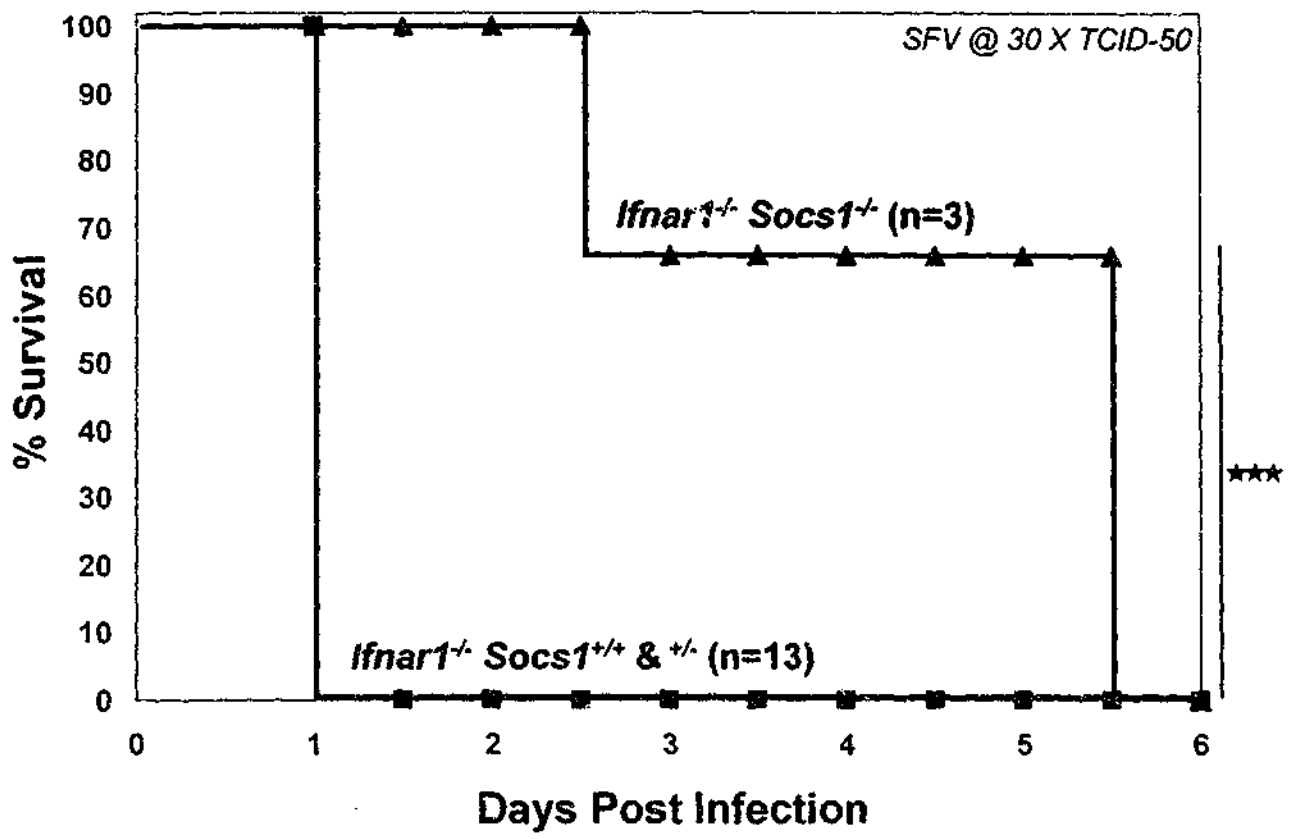


Figure 4.9: Deletion of the *Socs1* Gene in *Ifnar1*$^{-/-}$ Neonates Increases their Resistance to Viral Infection.

Survival plot measuring the resistance of SFV infected *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ and $^{+/-}$ -■- (n=13) and *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ -▲- (n=3) neonates aged between 5-10 days of age at 30 x TCID-50. The health of the neonates were subsequently monitored and fatalities recorded. Results demonstrated *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ and $^{+/-}$ neonates died within 24 hours while the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ neonates displayed a significantly increased resistance to viral infection surviving 5.5 days post infection as determined by a Logrank test (★★★P<0.001).

4.3 DISCUSSION

It has been demonstrated that the *Socs1*$^{-/-}$ neonates are more resistant to SFV infection compared to their wild-type and heterozygous littermates (Alexander *et al.*, 1999). This is consistent with the removal of an *in vivo* inhibitor of IFNs antiviral activity. Since both type I and II IFNs can elicit antiviral activity which SOCS1 can inhibit *in vitro* (Song & Shuai, 1998; Chapter 3), it was important to determine which IFN was responsible for SOCS1 regulation of the antiviral response observed in the *Socs1*$^{-/-}$ neonates. By using *in vivo* models, the experiments in this chapter have demonstrated that SOCS1 plays an important and critical regulatory role in type I IFN antiviral responses.

Several independent methods were used to achieve these findings. Firstly, viral replication was measured by viral titres in organs from virus infected *Socs1*$^{-/-}$, *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and wild-type neonates. These experiments demonstrated convincingly that 48 hours after infection there were significantly less viral particles present in the organs of the *Socs1*$^{-/-}$ neonates when compared to their wild-type and heterozygous littermates. This was due to a decrease in viral replication, implying an increase in viral particle clearance, rather than any other pro-survival mechanism. However this may have been a result of increased IFN signaling from higher concentrations of circulating IFN. Therefore, IFN levels were measured in the serum from these neonates. There was significantly less IFN present in the serum from *Socs1*$^{-/-}$ neonates 48 hours post infection. This result suggested that unlike the increase in IFNγ levels as measured by others (Marine *et al.*, 1999), the prolonged antiviral response to type I IFNs was due to an increased sensitivity to IFN. Therefore, the results presented from this chapter suggest that the *Socs1*$^{-/-}$ mice have a higher resistance level to SFV viral infection due to a hypersensitivity to the antiviral activity of IFN rather than increased production.

As both type I and II IFNs are capable of eliciting an antiviral response, the question still remained as to which IFN was responsible. The generation of the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice resulted in the removal of endogenous IFNγ, and the decreased survival of *Socs1*$^{-/-}$ mice, thereby produced a viable *in vivo* model

(Alexander *et al.*, 1999). This allowed for the effects of SOCS1 to be examined in the absence of IFNγ since this may have been masking roles other than neonatal death, that SOCS1 could be playing in other cytokine signaling systems, such as the type I IFN system. By utilising this model, the effects of type I IFNs and the source of the increased antiviral activity of *Socs1* removal examined. By studying both of these models (*Socs1*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$), the relative input into the antiviral response by both type I and II IFNs could be investigated.

The data presented here demonstrated that in the absence of SOCS1, an amplified type I IFN mediated antiviral activity occurred. This was demonstrated through viral infection experiments conducted on the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates with SFV that demonstrated resistance to infection. The viral titres from various organs were reduced following the same pattern as the *Socs1*$^{-/-}$ to viral infection. This result highlights that SOCS1 action is not specific to one organ/cell type. The resistance of *Ifng*$^{-/-}$ mice was no different to that of wild-type implying that IFNγ was not responsible for the amplified antiviral response. When anti-IFNα/β antibodies were administered to *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ neonates before SFV infection leaving these mice essentially defenceless against infection, results demonstrated the amplified antiviral response diminished. To further confirm these results, *Ifnar1*$^{-/-}$ and *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were infected with SFV to measure resistance. Previous studies have demonstrated that the *Ifnar1*$^{-/-}$ mice are highly susceptible to virus (Hwang *et al.*, 1995), therefore the results of these experiments demonstrated that removing *Socs1* increased resistance to infection, however they did eventually succumb to the lethal effects of the virus. Therefore by removing *Socs1*, essentially the 'brake' to type I IFN induced signaling, increased antiviral activity could be observed. This demonstrated that SOCS1 was acting as a critical regulator of the antiviral response elicited by type I IFNs.

There is evidence that SOCS1 plays a critical role in the regulation of IFNγ signaling (Starr *et al.*, 1998; Alexander *et al.*, 1999; Brysha *et al.*, 2001). The experiments in this chapter have demonstrated that SOCS1 also plays a critical

role in type I IFN signaling, specifically the antiviral signaling pathway. The implication of these findings is that after SFV infection, in the absence of *Socs1*, type I IFN antiviral activity results in decreased viral replication in most organs. This was not due to an increase in IFN levels. Therefore *Socs1* is affecting the type i IFN signaling pathway by allowing the amplification of an antiviral response resulting in protection from infection measured both by resistance to virus (increased % survival) and also in viral replication (decreased viral titres). Given the great potential of SOCS1 as an *in vivo* modifier of viral infection, it is important to understand the molecular mechanism whereby this is achieved. Therefore the mechanisms of how the type I IFN antiviral responses are amplified will be examined and discussed further in the following chapter.

CHAPTER 5

The Lack of *Socs1* Results in Sustained Type I IFN Signaling

5.1 INTRODUCTION

The *Socs1*$^{-/-}$ neonates have demonstrated an amplified antiviral response to SFV infection, which, through a series of *in vivo* experiments, was shown to result from type I IFN antiviral activity. Unlike IFNγ whose production is regulated by SOCS1 (Marine *et al.*, 1999), IFNα levels after viral infection did not appear to be changed in the absence of SOCS1. This is consistent with *in vitro* data on SOCS1 2ftgh overexpressing cells in which no change in IFN levels were observed (Chapter 3). These results then posed the question of how SOCS1 was regulating type I IFN signaling to achieve a sustained response to infection. This chapter will address the molecular mechanisms responsible for the amplified antiviral response to type I IFNs.

The SOCS proteins regulate cytokine signaling that occurs predominantly via the JAK/STAT pathway by either binding to activated JAK proteins or binding to cytokine receptors blocking STAT docking sites (Naka *et al.*, 1997; Endo *et al.*, 1997; Yasukawa *et al.*, 1999; Nicholson *et al.*, 1999). Binding of the SOCS proteins to their target proteins leads to ubiquitination and subsequent proteosomal degradation (Kamura *et al.*, 1998; Zhang *et al.*, 2001). SOCS1 can bind to each of the four JAK family members, rendering them inactive to phosphorylate the STATs thus inhibiting signal transduction (Naka *et al.*, 1997; Hilton *et al.*, 1998; Yasukawa *et al.*, 1999). SOCS1 binds to activated JAKs via its SH2 domain, however the KIR domain (24 amino acids immediately N-terminal of SH2 domain) is required for JAK kinase inhibition to occur (Nicholson *et al.*, 1999; Yasukawa *et al.*, 1999; Narazaki *et al.*, 1998). SOCS1 has been shown to extend IFNγ induced signaling (Brysha *et al.*, 2001). Cells from *Socs1*$^{-/-}$ mice show evidence of increased signaling by an increase in MHC class I measured both by immunohistochemistry and flow cytometry, an increase in type I and II IFN ISG expression and an increase in STAT1 activation by EMSA (Alexander *et al.*, 1999). Subsequent to these studies, prolonged activation of STAT1 phosphorylation was demonstrated after a pulse of IFNγ *in vitro* using primary hepatocytes and then *in vivo* (Brysha *et al.*, 2001). However IL6 signaling, was not regulated in the same manner (Brysha *et al.*, 2001), demonstrating that while SOCS1 is induced by and can inhibit a number

of cytokines *in vitro*, it has quite specific roles *in vivo*. This chapter will describe the mechanisms whereby SOCS1 regulates/inhibits type I IFN signaling.

Since both IFNα/β and IFNγ activate STAT1 phosphorylation after receptor engagement, this has been used as a measure of one of the earliest events in type I IFN signaling. Three type I IFN inducible genes have also been utilised as downstream readouts of the magnitude of this response. These genes are *Mx1*, *D3* and *2'-5' OAS*. *Mx1* encodes a 72 kDa protein that belongs to a small family of specifically IFNα/β responsive genes, which code for structurally related cytoplasmic and nuclear proteins (Sen, 2001). Constitutive expression of *Mx1* results in a high degree of resistance to the influenza virus, as *Mx1* possesses intrinsic antiviral activity (Zurcher *et al.*, 1992; Chapter 1). *D3* is a member of the mouse *Ifi200* gene cluster, which are a group of inducible nuclear proteins believed to have DNA binding capabilities that have been implicated in the control of cell proliferation and differentiation (Dawson & Trapani, 1996; Lembo *et al.*, 1998; Deschamps *et al.*, 2003). *D3* is inducible in macrophages by LPS and IFN (Tannenbaum *et al.*, 1993) and it is expressed in myeloid cells (Weiler *et al.*, 1999). *2'-5' OAS* is a type I IFN inducible gene which translates to a dsRNA dependent IFN induced enzyme that has intrinsic antiviral activity (Dong & Silverman, 1995; Ronni *et al.*, 1997; Chapter 1). The antiviral activity results from its ability to catalyse the synthesis of 2'-5' oligoadenylates from ATP. These 2'-5' oligoadenylates subsequently activate RNaseL which degrades viral RNA. Measurement of 2'-5' OAS activity has the advantage compared with mRNA measurements of ISGs, that the basal and induced enzyme activity can be quantitated accurately.

By using cells from the *Socs1*$^{-/-}$ mice different levels of the JAK/STAT pathway could be examined for potential mechanisms by which *Socs1* was able to effect the signaling, more specifically the antiviral signaling of type I IFN. In this chapter, three levels of the pathway are examined these being STAT1 phosphorylation, ISG expression by Northern blot and the protein activity of one ISG, 2'-5' oligoadenylate synthetase.

5.2 RESULTS

5.2.1 Kinetics of STAT1 Phosphorylation in *Socs1*$^{-/-}$ Cells

STAT1 activation was examined in response to IFNα4 in both *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice to determine whether the amplified response to type I IFNs was related to the levels or duration of STAT1 phosphorylation. Cell suspensions from spleen and lymph nodes were examined at 0, 10, 30, 60, 120 and 240 minutes after a 10 minute pulse of IFNα4 treatment for STAT1 tyrosine phosphorylation. In the spleen, there was no obvious difference in amplitude of the response with the peak phosphorylation reached 10 minutes after treatment in both *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ cells (Figure 5.1A). The duration of the response also did not appear to differ between the *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ cells as reduced phosphorylation was observed at 30 and 60 minutes and then were undetectable by 120 minutes (Figure 5.1A). Lymph nodes harvested from *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice had increased phosphorylated STAT1 levels at 10 minutes. Similar to the splenocytes, 60 minutes after stimulation phophorylated levels had decreased in both *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice (Figure 5.1B). Therefore even though the duration of STAT1 phosphorylation is lengthened in response to IFNγ in mice lacking *Socs1* (Brysha *et al.*, 2001), the results presented here demonstrate this is not observed with IFNα4 treatment.

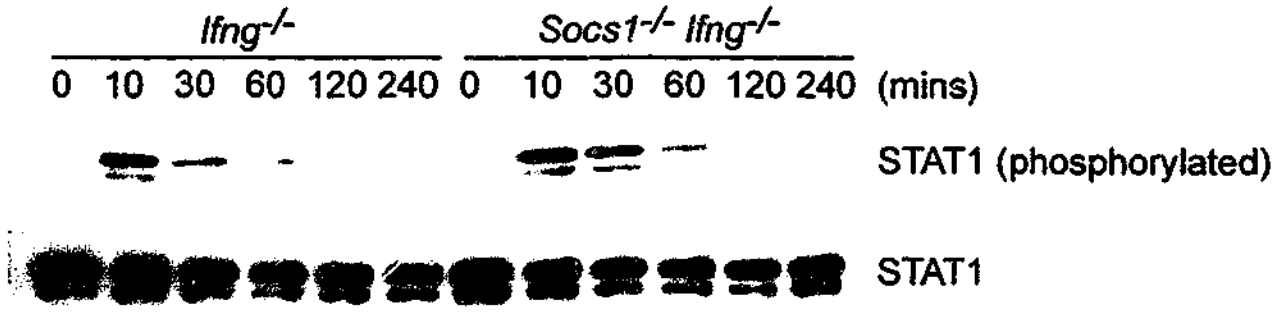
5.2.2 IFN Stimulated Genes *Mx1* and *D3* Display Unaltered Expression

In order to study the magnitude of a response to type I IFN, transcript levels of ISGs were measured in various organs isolated from wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice after *in vivo* treatment with IFNα4 for 0 and 4 hours. The *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice were used instead of the *Socs1*$^{-/-}$ mice due to the limited life

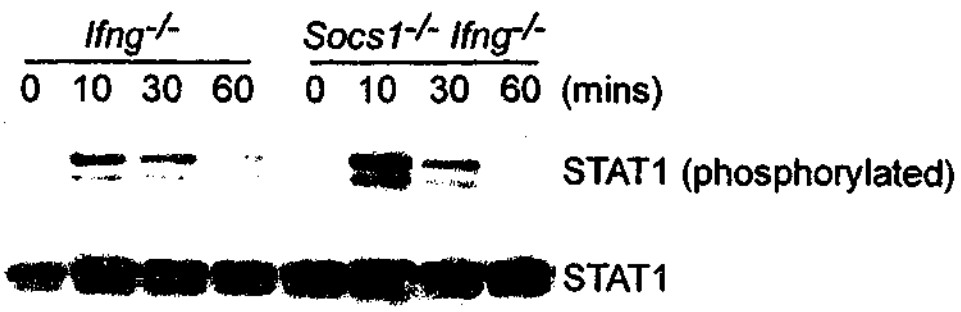
Figure 5.1: Phosphorylated STAT1 Levels are Unaltered in IFNα4 treated Splenocytes and Lymph Nodes from *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ Mice.

Western blots of IFNα4 treated cell lysates from the spleen and lymph nodes were prepared from *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and *Ifng*$^{-/-}$ mice. Protein lysates were electrophoresed on SDS PAGE gels and subsequently transferred onto immobilon membranes (refer to 2.4). Antibodies to both the phosphorylated and non-phosphorylated forms of STAT1 (STAT1α 91 kDa and STAT1β 84 kDa) were incubated with filters. Results demonstrate that there was no obvious difference in the levels of phosphorylated STAT1 in either splenocytes **A.** or lymph node cells **B.** in mice lacking SOCS1.

A. **Splenocytes**



B. **Lymph Nodes**



span of the latter, with the *Ifng*$^{-/-}$ mice used to control for the effects of no endogenous IFNγ.

The time point of 4 hours was chosen as induction of both *Mx1* and *D3* had been observed previously at this time (unpublished data). After culture, total RNA was extracted as described in section 2.3.2 and subsequently transferred onto membranes that were then hybridised with ^{32}P-labelled DNA fragments for *Mx1*, *D3* and *Gapdh* as a loading control (refer to 2.3.4). There was no apparent difference in the expression patterns of the 3.7 kb *Mx1* transcript 4 hours after treatment in the liver, thymus, kidney, spleen or lung between the three genotypes (Figure 5.2A). As the basal levels of the *Mx1* transcript were too low to quantitate as a fold induction, results were quantitated by expressing the induction as a ratio of *Mx1* expression:*Gapdh* expression. This analysis demonstrated that there was no difference in the magnitude of the response of either gene 4 hours after treatment (Figure 5.2B). Similarly with *D3* transcript levels, there appeared to be little difference between the wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ in the liver (Figure 5.3A). When induction levels were normalised to *Gapdh* and expressed as fold induction, *D3* was induced in the liver 10-fold, 11.5-fold and 10.8-fold in the wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ respectively (Figure 5.3B). These results imply that the ISGs *Mx1* and *D3* are not induced to different levels in the absence of *Socs1* 4 hours post IFNα4 treatment.

5.2.3 The Absence of *Socs1* Results in a Sustained IFNα/β Response

The third ISG to be examined in this chapter was 2'-5' OAS, which as previously mentioned is an IFN inducible enzyme that has intrinsic antiviral activity (Dong & Silverman, 1995; Ronni *et al.*, 1997). The activity of 2'-5' OAS can be measured accurately through the use of a radioactive enzyme assay that is sensitive enough to quantitate even low basal as well as inducible levels.

Murine embryo fibroblasts (MEFs) were isolated from wild-type, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ day 13-14 embryos (refer to section 2.5.3). MEFs were utilised for this

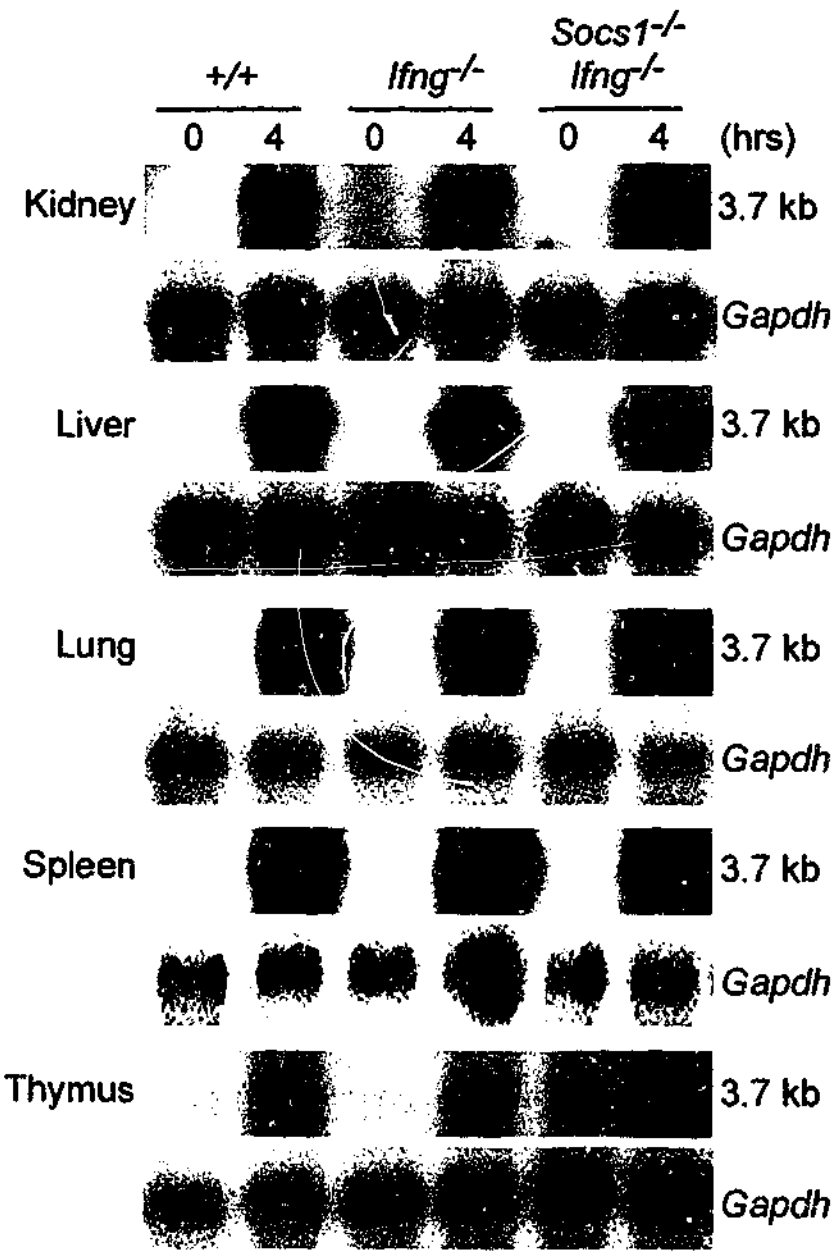
Figure 5.2: *Mx1* mRNA Expression levels in Wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ Mice.

Wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice were injected with IFNα4 for 4 hours *in vivo*. Mice, including control groups of each genotype were killed and various organs harvested for Northern blot analysis.

A. Northern blots of *Mx1* an IFN inducible gene, and *Gapdh* were examined across the three genotypes in kidney, liver, lung, spleen and thymus 4 hours after treatment.

B. Graphical representation of *Mx1* induced mRNA levels, expressed as a ratio to *Gapdh* mRNA levels. There was no difference in induced expression levels of *Mx1* 4 hours post IFNα4 treatment in the kidney ■, liver ■, lung ■, spleen ■ or thymus ■ of any of the three genotypes.

A.


+/+
Ifng-/-
Socs1-/- Ifng-/-
0 4 0 4 0 4 (hrs)
Kidney
3.7 kb
Gapdh
Liver
3.7 kb
Gapdh
Lung
3.7 kb
Gapdh
Spleen
3.7 kb
Gapdh
Thymus
3.7 kb
Gapdh

B.

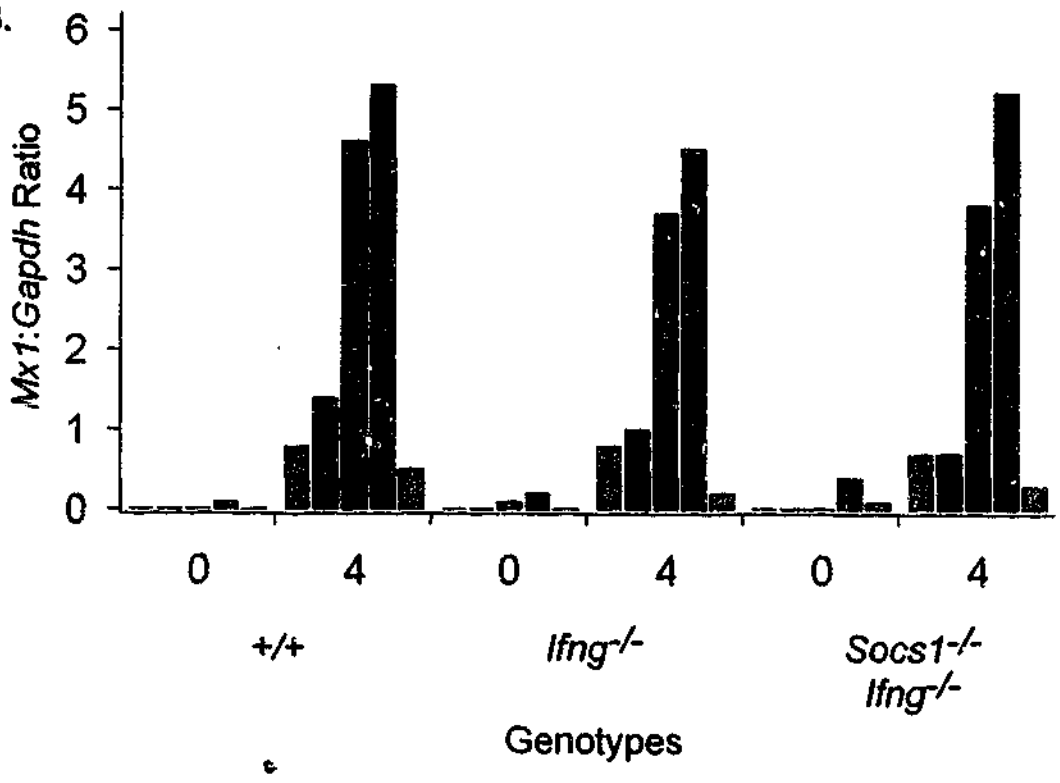

Mx1:Gapdh Ratio
0
1
2
3
4
5
6
0 4 0 4 0 4
+/+
Ifng-/-
Socs1-/- Ifng-/-
Genotypes

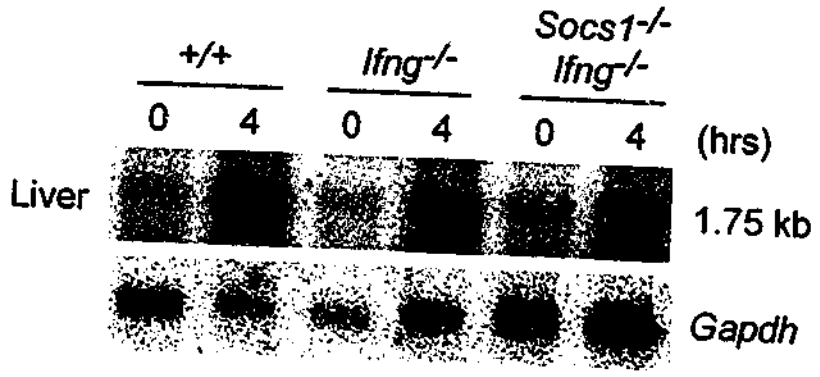
Figure 5.3: *D3* mRNA Expression levels in Wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ Mice.

Wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice were injected with IFNα4 for 4 hours *in vivo*. Mice, including control groups of each genotype were killed for Northern blot analysis.

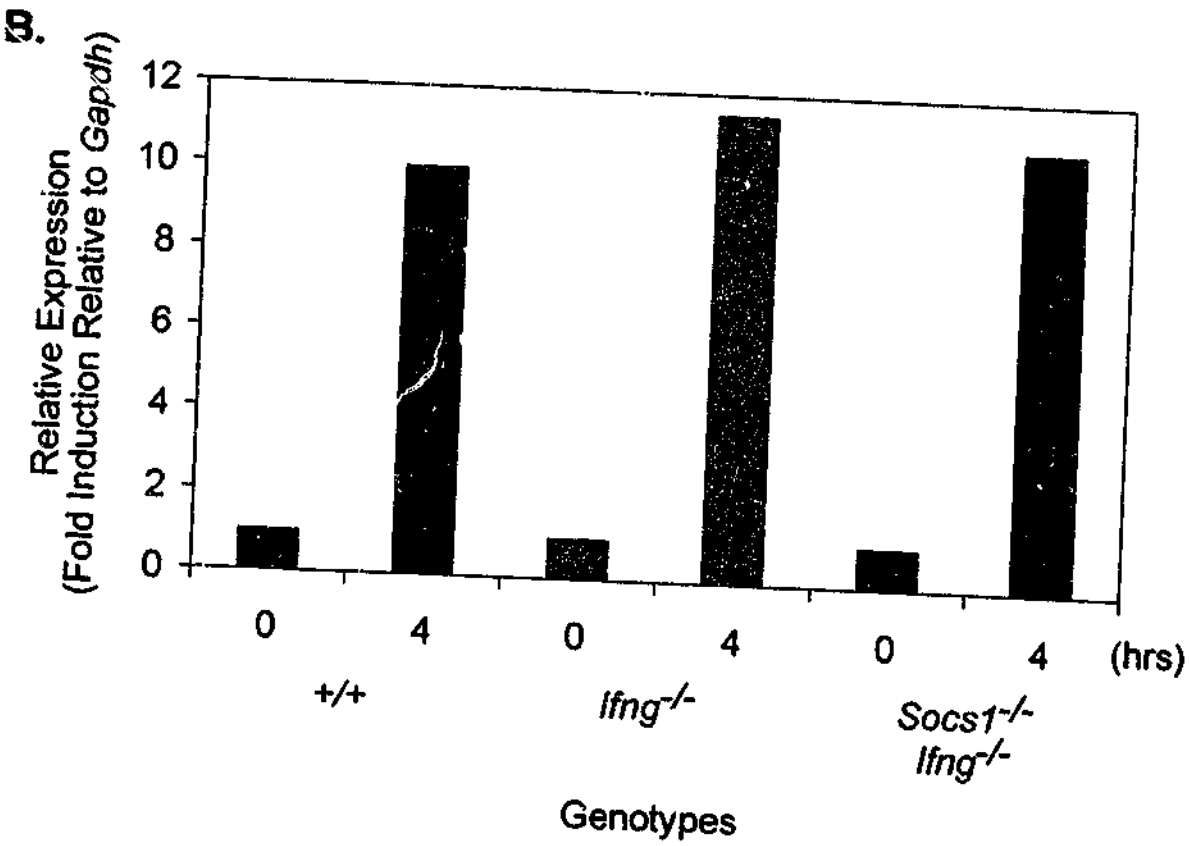
A. Northern blots of *D3*, an IFN inducible gene, and *Gapdh* in the liver before and a 4 hour treatment with IFNα4.

B. Induction levels were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. A 10-fold, 11.5-fold and 10.8-fold induction by IFNα4 after 4 hours was observed in livers from wild-type ◪, *Ifng*$^{-/-}$ ▩ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ ■ mice respectively.

A.


+/+
Ifng-/-
Socs1-/- Ifng-/-
0 4 0 4 0 4 (hrs)
Liver
1.75 kb
Gapdh

B.


Relative Expression
(Fold Induction Relative to Gapdh)
12
10
8
6
4
2
0
0 4 0 4 0 4 (hrs)
+/+
Ifng-/-
Socs1-/- Ifng-/-
Genotypes

assay to overcome the problem of the short life span of the *Socs1*$^{-/-}$ mice. These cells were then treated for 0, 24, 48, 72, 96, 120 and 144 hours with an initial 24 hour pulse of 1000 IU/ml of IFNα4 (refer to section 2.7). The mean basal levels +/- SEM obtained from this assay were 275.2+/-43.9, 286.9+/-30.16 and 356.4+/-35.27 mmoles/μg lysate in wild-type (n=3), *Socs1*$^{+/-}$ (n=6) and *Socs1*$^{-/-}$ (n=5) MEFs respectively. These values were not significantly different as measured by a Mann-Whitney Rank Sum test. The 2'-5' OAS activity in wild-type MEFs peaked after 72 hours of treatment with an enzyme activity of 17,980+/-3,460 mmoles/μg lysate, then had decreased to 7,065+/-3,142 mmoles/μg lysate 144 hours post treatment (Figure 5.4). In contrast 2'-5' OAS activity in *Socs1*$^{-/-}$ MEFs peaked after 48 hours of treatment with a similar activity of 16,250+/-1,090 mmoles/μg lysate. Importantly, the activity at 144 hours post treatment was maintained at a peak value of 17,160+/-1,822 mmoles/μg lysate (Figure 5.4). The *Socs1*$^{+/-}$ MEFs demonstrated an enzyme activity with a maximal increase of 16,180+/-1,531 mmoles/μg lysate 96 hours post treatment, then by 144 hours had decreased to 10,760+/-992.9 mmoles/μg lysate (Figure 5.4). This was not significantly different from the wild-type values, which is consistent with the heterozygous phenotypes and measured IFN responses (Starr *et al.*, 1998; Naka *et al.*, 1998; Chapter 4). The only statistically significant difference observed that was determined from a Mann-Whitney Rank Sum test in this assay was 144 hours post IFNα4 treatment, where the *Socs1*$^{-/-}$ MEFs displayed a sustained level of 2'-5' OAS enzyme activity compared to both *Socs1*$^{+/-}$ and wild-type MEFs (P<0.05). This represents conclusive data that basal ISG levels as well as the magnitude of response are unaltered by SOCS1, however the duration of the signal is sustained. Therefore it can be concluded that the lack of *Socs1* in these cells caused a prolonged increase of 2'-5' OAS activity.

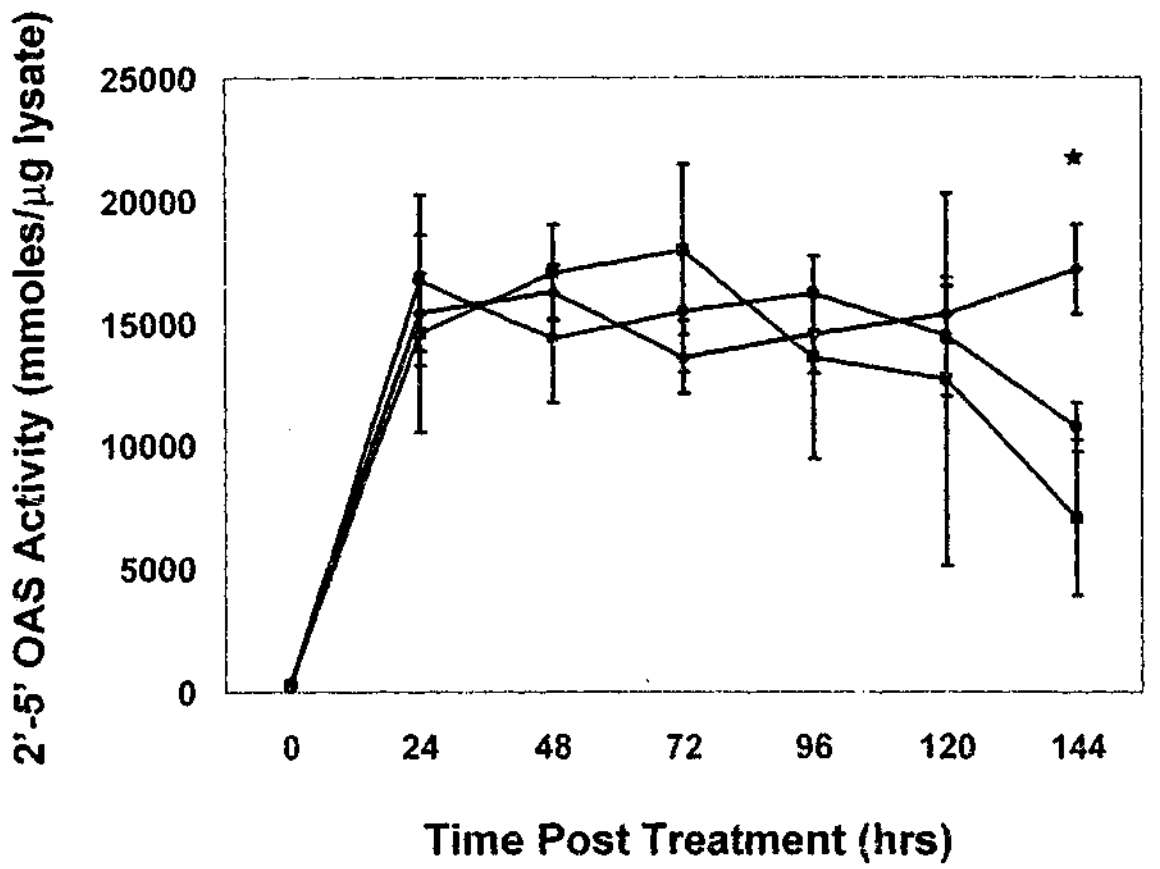


Figure 5.4: Duration of 2'-5' OAS Stimulation in Response to IFNα4.

Socs1$^{+/+}$ ■ (n=3), *Socs1*$^{+/-}$ ● (n=6) and *Socs1*$^{-/-}$ ◆ (n=5) day 13 murine embryo fibroblast cell lines were individually pulse treated with IFNα4 at 1000 IU/ml for 24 hours with lysates being collected every 24 hours up till 144 hours post initial treatment. Cells were lysed at 1 x 10^4 cells/μl of lysis buffer. Lysates were subsequently used in an enzyme assay which measures 2'-5' oligoadenylate synthetase activity in a radioactive readout. Cells lacking *Socs1* display a statistically significant sustained signal to mulFNα4 144 hours post treatment compared to the *Socs1*$^{+/+}$ and *Socs1*$^{+/-}$ cell lines which both display diminished signals to mulFNα4 at 144 hours (* P>0.05).

5.2.4 Resistance of Wild-type and *Socs1*$^{-/-}$ Murine Embryo Fibroblasts to Viral Infection

Since murine embryo fibroblasts demonstrated sustained 2'-5' OAS antiviral activity, these MEFs were used as target cells in CPE and CPE reduction assays (refer to section 2.5.6) to determine if the *Socs1*$^{-/-}$ cells were more sensitive to virus and to the protective effects of IFNα4. When wild-type and *Socs1*$^{-/-}$ cells were infected with SFV, there was no intrinsic difference in the potency of the virus in the absence of *Socs1* (Figure 5.5A). No statistical difference was detected by a Mann-Whitney Rank Sum test. This is consistent with SOCS1 overexpression data that showed SOCS1 had no direct effect on viral replication (Chapter 3).

When the cells were pretreated with different starting concentrations of IFNα4 (2500, 500 and 250 IU/ml) and then subsequently infected with SFV at 10 x TCID-50, the wild-type cells demonstrated lower viral titres than the *Socs1*$^{-/-}$ cells. The viral titres measured in the wild-type and *Socs1*$^{-/-}$ MEFs were 2.03+/-0.12 logs compared to 2.48+/-0.10 logs starting at 2500 U/ml respectively, 1.35+/-0.09 logs compared to 1.7+/-0.12 logs starting at 500 U/ml respectively and 0.95+/-0.14 logs compared to 1.23+/-0.10 logs starting at 250 U/ml respectively (Figure 5.5B). However these differences although consistent, were not significantly different as determined by Mann-Whitney Rank Sum tests. These results demonstrate that fibroblasts lacking *Socs1* have a tendency to be more sensitive than wild-type fibroblasts, but not to a significant extent. This data does somewhat conflict with findings from the *in vivo* experiments from chapter 4. The *in vivo* data demonstrated after 48 hours, viral titres were significantly lower in organs from the *Socs1*$^{-/-}$ by 3-logs. This could result from the small n values, where by more biological replicates could result in significance which would be consistent with *in vivo* data.

No difference in the amplitude of any response measured in experiments from the previous and current chapters has been observed, implying that the

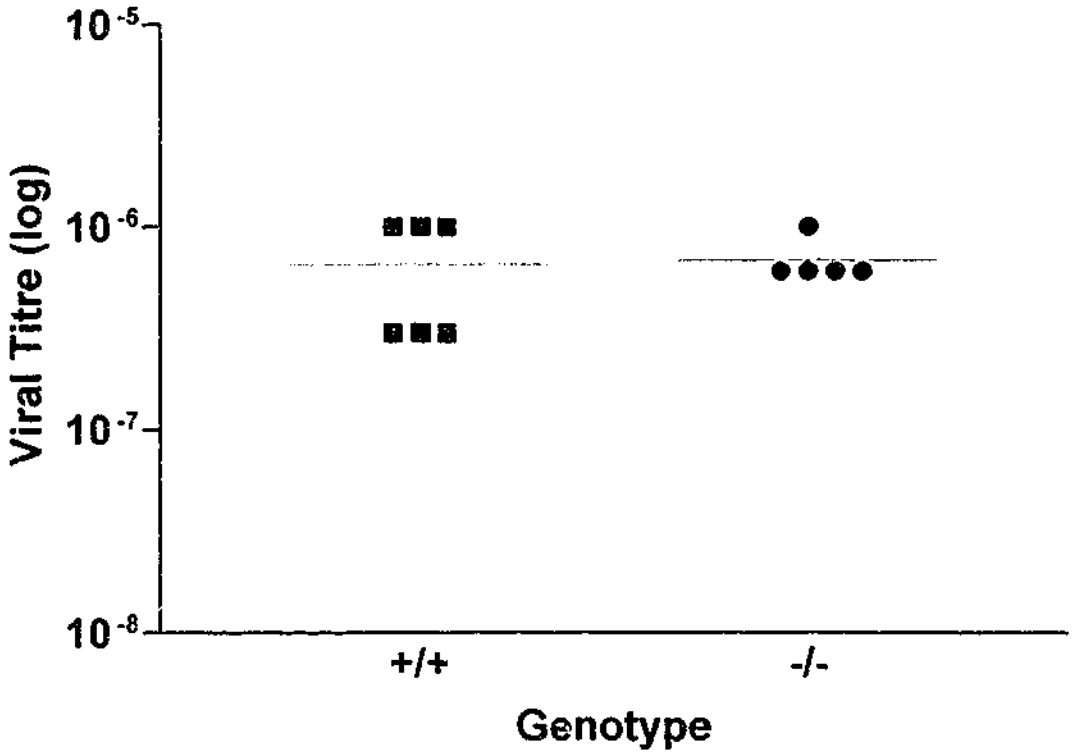
Figure 5.5: Resistance of Wild-type and *Socs1*$^{-/-}$ Murine Embryo Fibroblasts to SFV Viral Infection.

Wild-type and *Socs1*$^{-/-}$ murine embryo fibroblasts were generated from *Socs1*$^{+/-}$ matings in order to use as target cells in bioassays (refer to 2.5.6).

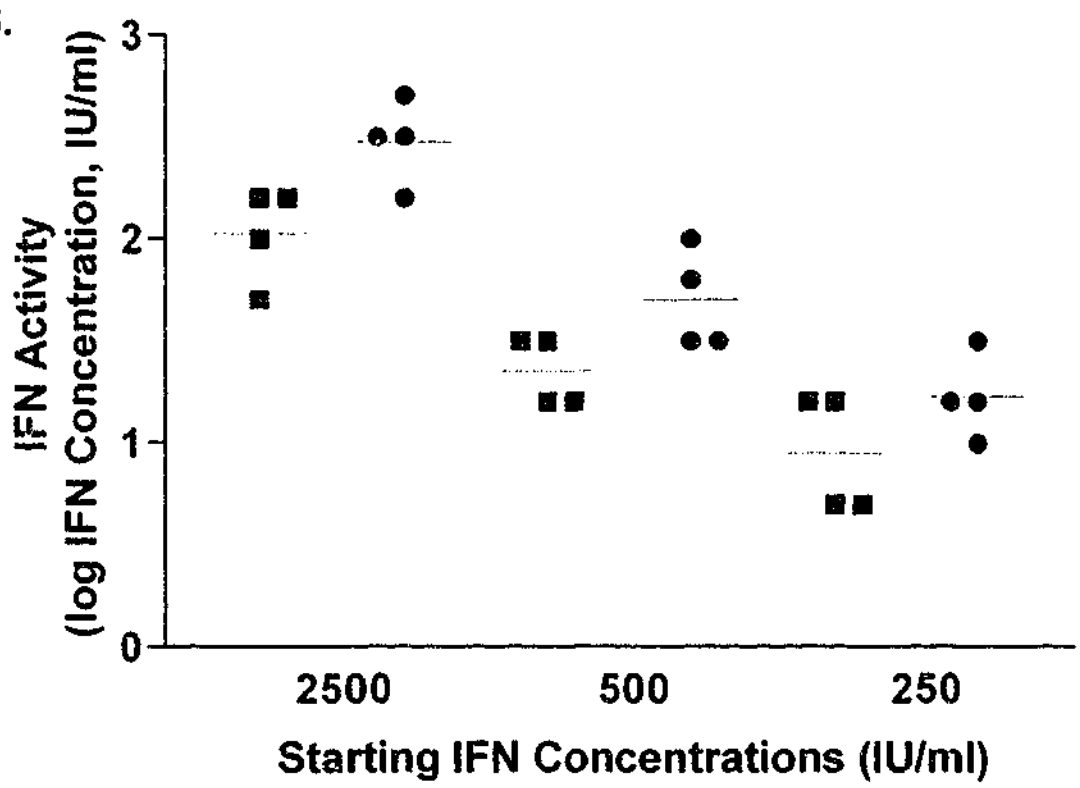
A. Wild-type ■ (n=6) and *Socs1*$^{-/-}$ ● (n=5) fibroblasts were treated with SFV at 10^{-2} and serially diluted in half logs in a CPE assay. Each point represents individual cell lines with the mean levels represented by the lines. Both wild-type and *Socs1*$^{-/-}$ cells had similar values for the TCID-50 which were not significantly different as determined by a Mann-Whitney Rank Sum test.

B. Wild-type ■ (n=4) and *Socs1*$^{-/-}$ ● (n=4) fibroblasts were treated with varying concentrations of IFNα4 prior to infection with SFV in CPE reduction assays in order to measure their antiviral activity. Wild-type cells demonstrated lower IFN titres than the *Socs1*$^{-/-}$ cells, however not significantly as determined by a Mann-Whitney Rank Sum test.

A.


Viral Titre (log)
10-5
10-6
10-7
10-8
+/+
-/-
Genotype

B.


IFN Activity
(log IFN Concentration, IU/ml)
3
2
1
0
2500
500
250
Starting IFN Concentrations (IU/ml)

increase in sensitivity to type I IFNs that has been observed is due to a sustained signal generated from the same level of IFN production.

5.3 DISCUSSION

The molecular mechanisms responsible for the amplified antiviral response observed in the *Socs1*$^{-/-}$ neonates in response to SFV infection, have been demonstrated to result from a sustained response to type I IFNs. This differs from IFNγ treatment, which results in a combination of an increase in strength and duration of a signal in *Socs1*$^{-/-}$ neonates (Alexander *et al.*, 1999; Brysha *et al.*, 2001). In contrast results throughout this chapter have demonstrated no difference in the magnitude of the responses to type I IFNs at least by a limited testing of ISGs.

STAT1 phosphorylation is one of the earliest events in signal transduction by a variety of cytokines such as IL2, IL3, IL6, IL10, IL11, IL12, IFNγ and IFNα/β (Larner *et al.*, 1993; Findbloom & Winestock, 1995; Leung *et al.*, 1995; Stahl *et al.*, 1995; Hou *et al.*, 1995; Meraz *et al.*, 1996). However the generation of the *Stat1*$^{-/-}$ mouse demonstrated that only IFN signaling was impaired suggesting that the lack of STAT1 was not as important *in vivo* as the *in vitro* data had indicated (Meraz *et al.*, 1996). STAT1 phosphorylation levels are prolonged in organs from *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice in response to IFNγ but not to IL6 suggesting specificity of SOCS1 regulation of IFNγ signaling *in vivo* (Brysha *et al.*, 2001). However similar to the lack of increased STAT1 phosphorylation in response to IL6, IFNα4 did not result in prolonged STAT1 phosphorylation in organs from the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice.

Alexander *et al.* (1999) demonstrated that ISGs *iNOS* and *Irf1* mRNA expression levels were elevated in various organs from *Socs1*$^{-/-}$ mice compared to wild-type controls. In response to IFNα4 treatment, no such effect was seen. *Mx1* and *D3* both displayed similar mRNA expression levels in organs from wild-type, *Ifng*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice after IFN injection. This again demonstrated that SOCS1 was not regulating IFNα/β signaling in the same manner as IFNγ signaling.

Measuring the basal levels of 2'-5' OAS enzyme demonstrated that basal levels did not differ between wild-type, *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ cells suggesting cells

were not 'primed' to elicit an amplified response in the absence of SOCS1. This contrasts with the decrease in basal 2'-5' OAS observed with removal of the constitutive IFN response in *Ifnar1*$^{-/-}$ mice (Hwang *et al.*, 1995). Perhaps the basal level that exists in normal mice cannot be further increased by de-repression of IFN signals by the removal of SOCS1. After IFNα4 stimulation, results of 2'-5' OAS activity in wild-type cells increased to 16,000 mmoles/μg lysate by 72 hours then reduced again towards basal levels by 144 hours. However interestingly 2'-5' OAS enzyme activity in cells isolated from *Socs1*$^{-/-}$ embryos demonstrated a signal that increased to the same intensity as the wild-type and was still sustained at this level 144 hours after initial stimulation. Therefore it could be concluded that like IFNγ signaling, IFNα/β responses were sustained, however unlike IFNγ signaling, there was no increase in the magnitude of the response to IFNα4 (Brysha *et al.*, 2001).

SOCS1 regulates type I IFN production via a different mechanism to IFNγ (Alexander *et al.*, 1999; Brysha *et al.*, 2001). This may be due to the cytokine based production of IFNγ (Sen, 2001) that is SOCS regulated, i.e. IL12. Whereas IFNα/β is induced by virus directly which therefore may not be directly regulated by SOCS. IFNγ levels are increased in *Socs1*$^{-/-}$ mice (Marine *et al.*, 1999), however IFNα/β levels are not (Chapter 4), which implys that an increased response to type I IFNs is not due to increased levels of circulating type I IFN. Therefore, the mechanism that appears to be regulating type I IFN signaling is the lack of SOCS1 in these mice to switch off or dampen the signal from the receptor complex resulting in the prolongation of IFNα/β signaling. The lack of STAT1 tyrosine phosphorylation appears to contradict this hypothesis, but the results from the 2'-5' OAS assays clearly demonstrate a sustained response to IFNα4 treatment that is not seen in either wild-type or *Socs1*$^{+/-}$ cells under the same conditions. Therefore from these results it suggests that tyrosine phosphorylated STAT1 mediated signaling is not responsible for the sustained type I IFN induced 2'-5' OAS activity. However further studies into the STAT1 tyrosine phosphorylation may demonstrate differences in the duration of phosphorylation between 60 and 120 minutes with more time points examined. STAT1 serine phosphorylation may be responsible or rather STAT-

independent JAK dependent pathway may be the cause of the sustained 2'-5' OAS activity observed. However further studies into the STAT1 phosphorylation could demonstrate that STAT1 serine phosphorylation may be responsible or rather a STAT-independent JAK dependent pathway may be the cause.

The results from both this chapter and the previous have demonstrated both *in vivo* and *in vitro* that *Socs1* plays a critical role in the antiviral responses elicited by type I IFNs. The observation that the *Socs1*$^{-/-}$ mice demonstrated a higher resistance to SFV infection has been shown to result from a sustained and exclusive response to type I IFN while the magnitude of the response is apparently unaltered. Under normal physiological conditions, SOCS1 decreases the duration of ISG inductions resulting in decreased biological effect, hence working as a negative regulator of type I IFN signaling. In order to further elucidate the interaction of SOCS1 with type I IFN signaling *in vivo*, the *Socs1*$^{-/-}$ mice can be crossed with other mouse lines with specific defects in type I IFN signaling.

CHAPTER 6

Type I IFNs Contribute to SOCS1-Mediated Physiological and Pathological Effects

6.1 INTRODUCTION

SOCS1 has been demonstrated *in vitro* to be induced by type I IFNs, as well as inhibit their actions (Chapter 3). *In vivo* experiments demonstrated that the removal of SOCS1 caused an enhanced type I IFN response to virus (Chapter 4). This was then demonstrated to result from a sustained response to type I IFNs (Chapter 5). Therefore it could be concluded that SOCS1 was playing an important role in the regulation of type I IFN signaling of antiviral effects. While SOCS1 was shown to decrease the duration but not amplitude of signaling in the absence of sustained STAT1 tyrosine phosphorylation, the question of how this was achieved arose. In order to further understand the mechanism and breadth of SOCS1 effects on type I IFN signaling, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mouse models were crossed with *Socs1*$^{-/-}$ mice to examine IFNAR receptor-chain specific effects on signaling.

In order to study the functions of the type I IFN system, *in vivo* knockout mouse models *Ifnar1*$^{-/-}$ (Müller *et al.*, 1994; Hwang *et al.*, 1995) and *Ifnar2*$^{-/-}$ (Hertzog, unpublished) have been generated in our lab. Extensive analysis of the haematopoietic lineages of each of the single *Ifnar*$^{-/-}$ mice have generated data to demonstrate that the two receptor knockout models present with slightly different phenotypes. The *Ifnar1*$^{-/-}$ mice are ill equipped to generate antiviral or antiproliferative effects in response to exogenous IFNα/β (Hwang *et al.*, 1995). Haematopoietic cells of myeloid lineage were increased in number, while all other parameters of haematopoiesis were comparable to wild-type mice. Interestingly, the *Ifnar2*$^{-/-}$ mice were demonstrated to be highly susceptible to viral infection, albeit not to the same extent as the *Ifnar1*$^{-/-}$, while the abnormalities found in these mice were an increased thymus size with an increase in $CD8^+$ cells. This resulted from a malfunction in the apoptotic signaling of these cells within the thymus (Hertzog, unpublished). Similar to the *Ifnar1*$^{-/-}$ mice, all other parameters of haematopoiesis were normal in the *Ifnar2*$^{-/-}$ mice. The implication from these studies was that in the case of each *Ifnar*$^{-/-}$, the remaining chain, IFNAR2 or IFNAR1 with their associated JAK1 or TYK2 respectively, was capable of transducing a limited signal. Therefore crossing *Socs1*$^{-/-}$ mice with the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ models had a dual utility. Firstly it

could elucidate how SOCS1 was acting on the proximal type I IFN signaling apparatus. Secondly it could provide amplification of any limited residual signaling in either of the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ mice and facilitate mechanistic studies.

The removal of endogenous IFNγ from the *Socs1*$^{-/-}$ mice by the generation of the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mouse demonstrated that the lethality of the *Socs1*$^{-/-}$ was due to a hypersensitivity to IFNγ (Alexander *et al.*, 1999). Similarly by removing either *Ifnar1* or *Ifnar2* from the *Socs1*$^{-/-}$ mice by virtue of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ and the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$, any effects of type I IFN on the *Socs1*$^{-/-}$ mice or specific phenotypic features of these mice could be elucidated.

6.2 RESULTS

6.2.1 Establishment of *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ and *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ Mouse Lines

In order to study in more detail the effects that *Socs1* was having in the type I IFN system, *Ifnar1*$^{-/-}$ and *Socs1*$^{+/-}$ mice were bred to obtain *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ mice that were subsequently interbred to obtain *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice. Similarly, *Ifnar2*$^{-/-}$ and *Socs1*$^{+/-}$ mice were bred to obtain *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ mice which were subsequently interbred to obtain *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice. Both colonies were maintained by breeding double heterozygous mice in conventional housing. Due to the heterozygous matings, all mice were required to be genotyped for the status of *Socs1*, as described in chapter 4, then depending on which colony were genotyped for either *Ifnar1* or *Ifnar2*.

Genomic DNA extracted from the tails of neonatal mice that were screened for *Ifnar1* allele status was digested with *Eco*RI. The digested DNA was electrophoresed through 1% agarose gels and transferred to Genescreen Plus membranes (refer to 2.2.3.2). The membranes were hybridised with a ^{32}P-labelled 1.7 kb *Bam*HI/*Sac*I fragment of the *Ifnar1* gene (refer to 2.2.2). The wild-type *Ifnar1* allele was detected at the expected size of 3.8 kb, and the targeted allele at 4.8 kb (Figure 6.1A).

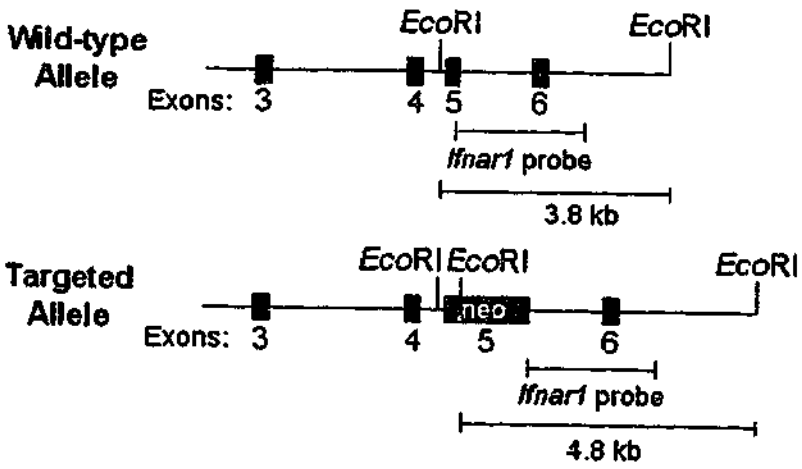
Genomic DNA that was screened for *Ifnar2* allele status was extracted from the tails of neonatal mice and subsequently used in a PCR reaction as a template (refer to 2.2.3.3). Three positive controls of known genotype were included as well as one negative control containing no DNA. PCR products were subsequently visualised on a 1% agarose gel. The wild-type *Ifnar2* allele was detected at the expected size of 1.3 kb, and the targeted allele at 0.15 kb (Figure 6.1B).

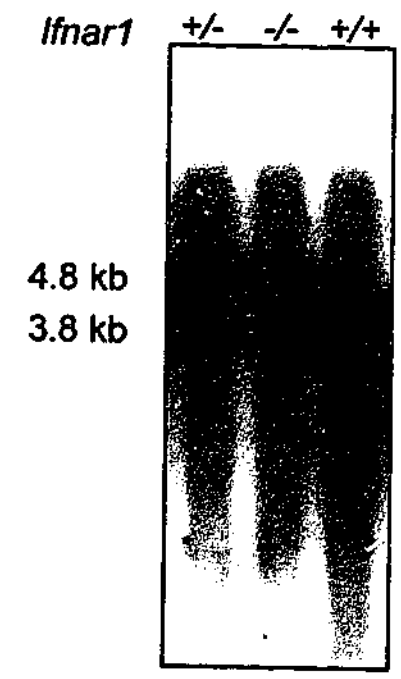
Figure 6.1: Genotyping of Neonatal Mice with a Targeted Mutation of the *Ifnar1* gene and the *Ifnar2* gene.

A. A schematic representation of the targeting strategy of the *Ifnar1* allele. The targeted *Ifnar1* allele was disrupted with the insertion of a *neo* gene into exon 5. Neonates produced from *Ifnar1*$^{+/-}$ matings were genotyped by Southern hybridisation techniques using a 1.7 kb *Bam*HI/*Sac*I fragment (refer to 2.2.2). The expected band sizes were 3.8 kb and 4.8 kb for the wild-type and *Ifnar1*$^{-/-}$ alleles respectively.

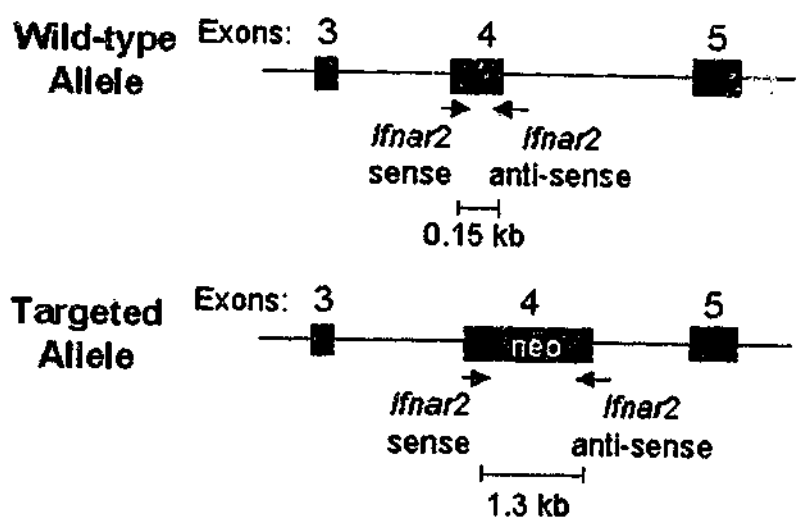
B. A schematic representation of the targeting strategy of the *Ifnar2* allele. The targeted *Ifnar2* allele was disrupted with the insertion of a *neo* gene into exon 4. Neonates generated from *Ifnar2*$^{+/-}$ matings were genotyped by polymerase chain reaction (refer to 2.2.3.3). The expected band sizes were 1.3 kb and 0.15 kb for the wild-type and *Ifnar2*$^{-/-}$ alleles respectively.

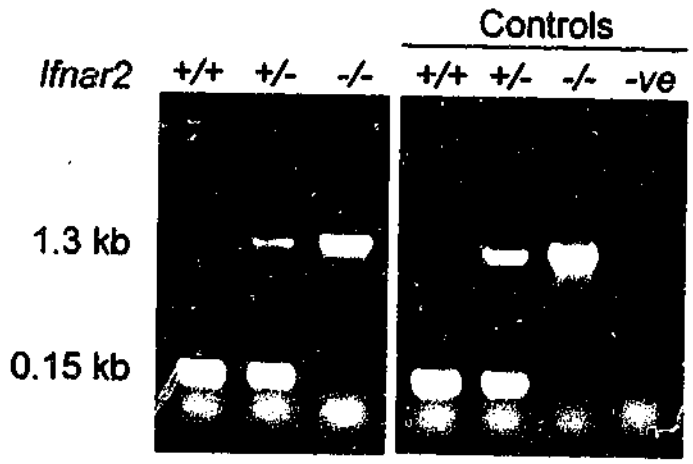
A.


Wild-type Allele
EcoRI
EcoRI
Exons: 3 4 5 6
Ifnar1 probe
3.8 kb
Targeted Allele
EcoRI EcoRI
EcoRI
neo
Exons: 3 4 5 6
Ifnar1 probe
4.8 kb


Ifnar1
+/- -/- +/+
4.8 kb
3.8 kb

B.


Wild-type Allele
Exons: 3 4 5
Ifnar2 sense
Ifnar2 anti-sense
0.15 kb
Targeted Allele
Exons: 3 4 5
neo
Ifnar2 sense
Ifnar2 anti-sense
1.3 kb


Controls
Ifnar2
+/+ +/- -/- +/+ +/- -/- -ve
1.3 kb
0.15 kb

6.2.2 *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ Breeding Generates the Expected Genotypes in Mendelian Frequencies

Ifnar1$^{+/-}$ *Socs1*$^{+/-}$ mice were interbred in conventional housing to generate mice that were null for both *Ifnar1* and *Socs1*. From these breedings there could result 9 possible genotypes that were obtained and observed in Mendelian frequencies ranging from 1-4/16 (Table 6.1). Out of the 219 pups that were genotyped, there were an additional 10 pups whose births were recorded, however not genotyped but died before 21 days of age. A χ^2 test was performed from the observed and expected numbers without the extra 10 pups. Results demonstrated that the 9 possible genotypes were significantly different from expected Mendelian frequencies (P<0.05). Table 6.1 illustrates that the observed mice numbers of *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ and *Ifnar1*$^{+/-}$ *Socs1*$^{-/-}$ were lower than expected. The 10 pups not genotyped all died prior to 3 weeks of age making them likely to be *Socs1*$^{-/-}$ mice. If these were redistributed in a 2:1 ratio into *Ifnar1*$^{+/-}$ *Socs1*$^{-/-}$ or *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ groups, the observed numbers changed to be closer to the expected numbers for these two genotypes. This redistribution decreased the χ^2 value below the P=0.05 value to except the hypothesis that the 9 possible genotypes from the *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ matings were not significantly different from the expected Mendelian frequencies (P>0.05).

The survivals of each of the 9 possible genotypes were graphed against time in weeks post birth. Results demonstrate that the life spans of the *Socs1*$^{-/-}$ mice were affected by the status of the *Ifnar1* allele (Figure 6.2). This figure demonstrates that heterozygous alleles for both or either one of *Ifnar1* or *Socs1* had no effect on the survival of mice exhibiting 94-100% survival. *Ifnar1*$^{-/-}$ mice usually have a slightly lower survival rate as expected, as they are more prone to infection (Müller *et al.*, 1994; Hwang *et al.*, 1995). This study demonstrated at 26 weeks of age that the *Ifnar1*$^{-/-}$ mice had an 82% survival rate compared to the 100% of the wild-type mice, which was not significantly different as determined by a Logrank test. However the lack of one copy of the *Socs1* allele in the *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ mice reduced the survival further to 68% by 26 weeks of

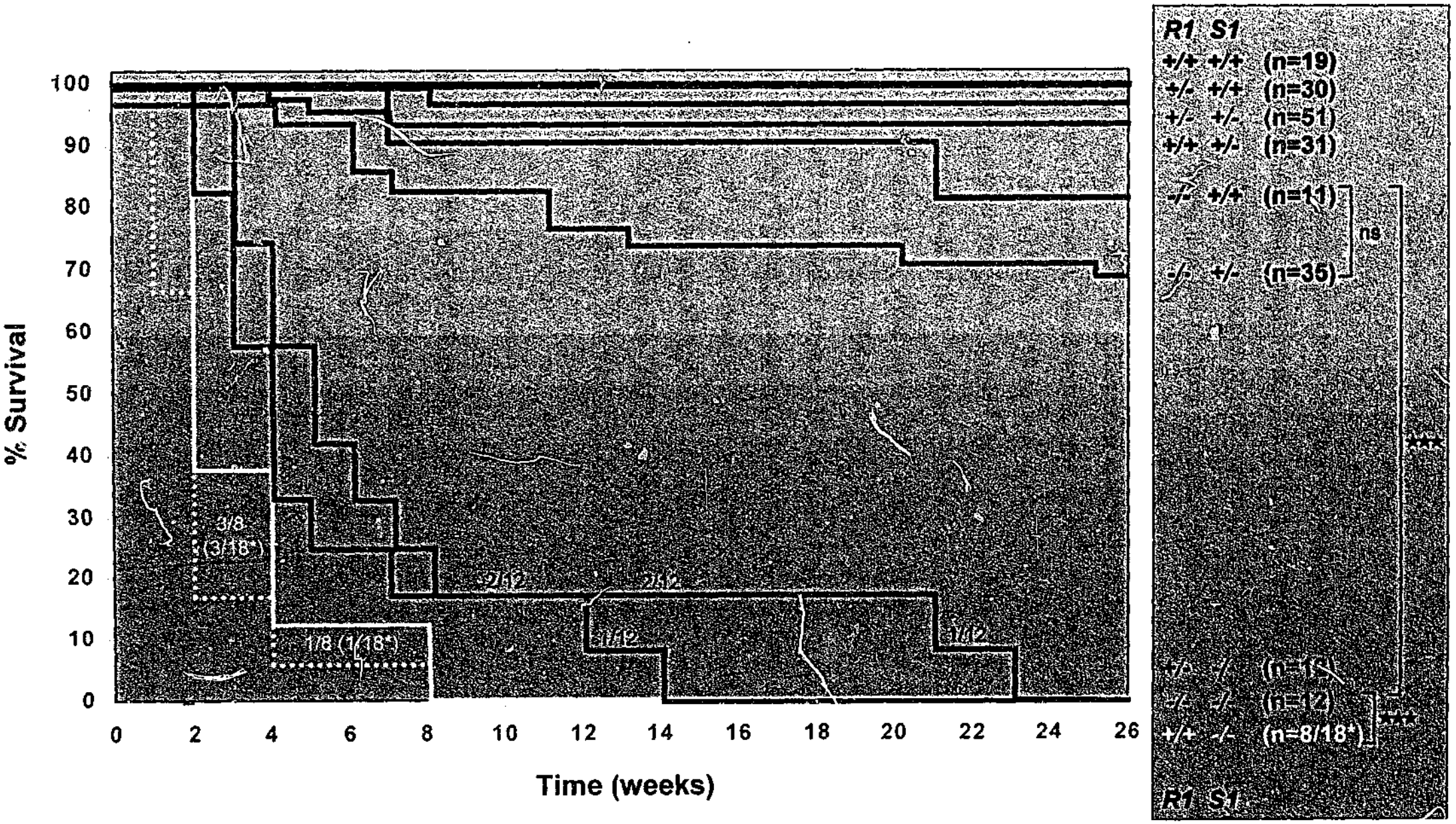
Table 6.1: Frequency of Expected Genotypes from *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ Matings.

From double heterozygous matings, each of the 9 possible genotypes were observed in Mendelian frequencies when the additional pups are redistributed (χ^2=9.94<P=0.05, df=8) (P>0.05). An additional 10 pups were recorded, however not genotyped as they died before 21 days. With the observed numbers appearing lower in two genotypes □ these 10 pups most likely were of either *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ or *Ifnar1*$^{+/-}$ *Socs1*$^{-/-}$ genotype.

		Number of mice from *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ crosses	
Ifnar1	*Socs1*	Expected numbers	Observed numbers
+/+	+/+	14	19
+/+	+/-	27	31
+/+	-/-	14	8
+/-	+/+	27	30
+/-	+/-	55	51
+/-	-/-	27	12
-/-	+/+	14	11
-/-	+/-	27	35
-/-	-/-	14	12
Deaths not genotyped			10
TOTALS:		219	219

Figure 6.2: Survival of Mice Generated from *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ Crosses.

From *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ X *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ breeding, each of the 9 possible genotypes were observed in Mendelian frequencies. Heterozygote alleles for both or either one of *Ifnar1* and *Socs1* had no effect on the survival of mice housed under conventional conditions. *Socs1*$^{-/-}$ mice suffer neonatal death by 3 weeks due to a hypersensitivity to IFNγ, however 3/8 (3/18*) mice survived to 4 weeks and 1/8 (1/18*) survived to 8 weeks. Out of 12 *Ifnar1*$^{+/-}$ *Socs1*$^{-/-}$ —— mice, 2/12 survived 7 weeks, by 21 weeks 1/12 mice were alive which survived to 23 weeks of age. Out of 12 *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ —— mice 2 survived to 8 weeks, by 12 weeks 1 had survived which died at 14 weeks of age. All statistics were determined by Logrank tests (***P<0.001).


% Survival
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (weeks)
3/8 (3/18*)
1/8 (1/18*)
2/12
2/12
1/12
1/12
R1 S1
+/+ +/+ (n=19)
+/- +/+ (n=30)
+/- +/- (n=51)
+/+ +/- (n=31)
-/- +/+ (n=11)
ns
-/- +/- (n=35)
***
+/+ -/- (n=16)
-/- -/- (n=12)
+/+ -/- (n=8/18*)
***
R1 S1

age. This was significantly different from the wild-type mice ($P<0.01$) but not from the *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$.

Previous studies have shown that the *Socs1*$^{-/-}$ mice die at 3 weeks of age (Starr *et al.*, 1998). However, results from these studies demonstrated that 38% (3/8) of the *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ mice genotyped survived beyond 3 weeks of age. If the additional 10 pups that died before weaning were *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ then this percentage would have been reduced to 17% (3/18) survival at 3 weeks of age. In contrast *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice demonstrated 100% (12/12) survival at 3 weeks of age (weaning). By 4 weeks this number had dropped to 75% (9/12) as opposed to the 17/38% (3/18 or 3/8) survival of the *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ mice. By 5 weeks 58% (7/12) of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were still alive compared to 5-13% (1/18 or 1/8) of *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ mice. By 8 weeks none of the *Ifnar1*$^{+/+}$ *Socs1*$^{-/-}$ mice were alive, while 25% (3/12) *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were surviving, which was statistically significant ($P<0.001$). By 14 weeks 8% (1/12) of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were alive which died one week later. Interestingly mice of *Ifnar1*$^{+/-}$ *Socs1*$^{-/-}$ genotype started to become sick and die one week before the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice, however 17% (2/12) mice survived 21 weeks with 8% (1/12) living till 23 weeks of age. This suggests that the lack of one allele of *Ifnar1* is enough to enable the *Socs1*$^{-/-}$ mice to survive past weaning. These results suggest that *Ifnar1* is contributing to the neonatal lethality of the *Socs1*$^{-/-}$ mice.

6.2.3 Postnatal Lethality and Histopathology of *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ Mice

At birth *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice are not observably distinct from any of the other 8 possible genotypes. As they get older, by 4 weeks they can generally be identified by partly or completely closed eyes and are usually smaller in size (Figure 6.3A). At 4 weeks of age the average weight +/- SEM of *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ female mice (n=3) was 9.2+/-0.15 g compared to 15.8+/-0.40 g of wild-type females (n=5), 16.73+/-1.14 g of *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ females (n=6) and 16.98+/-0.81 g of *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ females (n=11) (Figure 6.3B). Mann-Whitney Rank Sum tests demonstrated that the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ female mice weighed

Figure 6.3A: Appearance of Mice at 3-4 weeks from *Ifnar1*$^{+/-}$ *Socs1*$^{+/-}$ crosses.

Ifnar1$^{-/-}$ *Socs1*$^{-/-}$ mice appear smaller in size at 3-4 weeks than their *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ littermate controls.

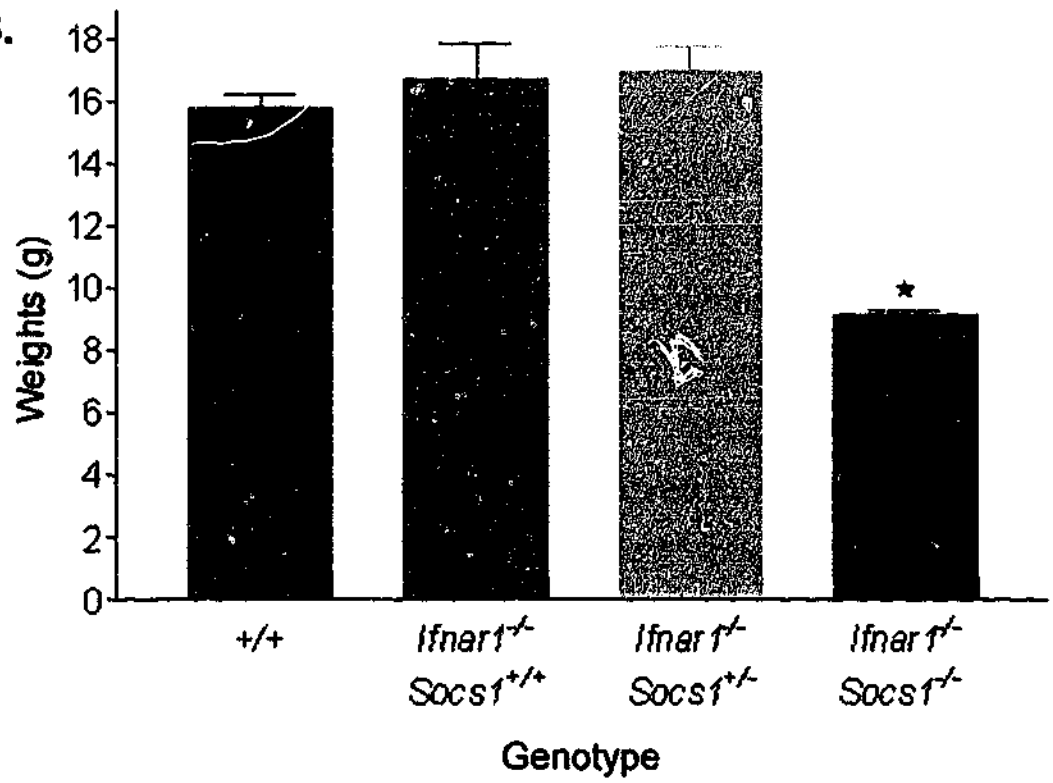
Figure 6.3B: *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ Mice Weigh less than their *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$, *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ and wild-type littermates.

Ifnar1$^{-/-}$ *Socs1*$^{-/-}$ ■ (n=3) mice weigh less than their *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ ■ (n=6), *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ ▩ (n=6) and wild-type ■ (n=5) littermates at 4 weeks of age ($\star P<0.05$).

A.


+/+
Ifnar1-/- Socs1-/-

B.


Weights (g)
0
2
4
6
8
10
12
14
16
18
*
+/+
Ifnar1-/- Socs1+/+
Ifnar1+/- Socs1+/-
Ifnar1+/- Socs1-/-
Genotype

significantly less than either wild-type, *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ or *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ females (P<0.05).

The *Socs1*$^{-/-}$ mice have a much smaller body size from 10 days of age than their wild-type littermates (Starr *et al.*, 1998), in contrast the body size differences of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice are not observable until 3-4 weeks of age. In order to determine the histopathological changes in these mice, *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$, *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ and *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were killed at 6-8 weeks to harvest organs/tissue (refer to section 2.6.3) for histological examination. Results were compiled by Professor Donald Metcalf group in the Division of Cancer and Haematology at the Walter and Eliza Hall Institute. An extensive analysis of the histopathology of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice compared to the *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ was completed examining the liver, thymus, heart, kidney, spleen, lung, pancreas, salivary glands, bladder, muscle, eyes, marrow, gut, skin and lymph nodes (Summarised in Table 6.2). Results showed that there were obvious changes in the muscle, which sections showed severe infiltration by T lymphocytes, macrophages and eosinophils. The heart suffered infiltration by similar cells while the eye displayed corneal infiltration and ulceration (Summarised in Table 6.2).

The cornea in the *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ is normal diplaying no infiltration (Figure 6.4A), however the cornea of *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice is infiltrated by mononuclear cells with granulocytes present in the iris and anterior chamber (Figure 6.4B). Skeletal muscle was infiltrated by T cells, macrophages and eosinophils with damage to the muscle cells in the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice (Figure 6.4D) contrasting to the normal skeletal muscle observed in the *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ mice (Figure 6.4C). The lungs of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were infiltrated with T cells and macrophages resulting in thickening of the alveolar walls (Figure 6.4F) which was not apparent in the *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ mice (Figure 6.4E). Histology of the salivary gland (Figure 6.4G) and the bone marrow (Figure 6.4H) also demonstrated infiltration of lymphoid cells and an excess proportion of neutrophilic granulocytes respectively in the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice. These mice demonstrate similar pathological features to the *Socs1*$^{-/-}$ *Ifng*$^{+/-}$ mice. This suggests that the loss of IFNAR1 and therefore a loss of efficient type I IFN

Table 6.2: Pathology of *Ifnar1 Socs1* Mice.

	Pathology	Percent Frequency	
		Ifnar1$^{-/-}$ *Socs1*$^{+/+}$ or *Ifnar1*$^{-/-}$ *Socs1*$^{+/-}$ mice (n=28)	*Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ (n=14)
Liver	-necrosis	9	21
	-fatty degeneration	25	29
	-haematopoietic foci - excess	71	57
	-lymphoid foci	4	21
	-plasma cells	4	7
	-Kupffer cells excess	32	50
Thymus	-cortical atrophy	36	100
	-medulla enlargement	7	11
Heart	-massive infiltration	0	0
	-infiltration	4	71
Kidney	-lymphoid infiltration	7	7
	-immaturity	25	14
Spleen	-large follicles	52	57
	-small follicles	32	29
	-germinal centres	39	69
	-excess erythropoiesis	79	93
	-plasma cells	0	0
Lung	-lymphoid follicles	11	79
	-plasma cells	4	21
	-granulocyte-macrophage infiltration	50	43
Pancreas	-normal	68	36
	-lymphocyte infiltration	14	36
	-acinar damage	18	36
	-macrophage infiltration	0	36
Salivary Glands	-lymphoid foci	4	21
Bladder	-lymphoid foci	0	0
	-granuloma	0	14
Muscle	-severe infiltration	0	57
	-minor macrophage infiltration	14	29
Eyes	-corneal infiltration	0	71
	-ulceration	0	7
	-destruction	0	0
Marrow	-G>M (Eo+)	46	86
Gut	-granuloma	0	0
Skin	-normal	89	14
	-patches of E + keratin	11	71
Lymph Node	-excess plasma cells	0	26
Reticulum Cell Hyperplasia		18	36

Figure 6.4: Pathology of *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ Mice and *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ Mice.

Mice ranging in age from 4-6 weeks of age were sacrificed and organs/tissues/bones were harvested and fixed in 10% formalin. Sections were stained with haematoxylin and eosin.

A. Cornea, iris and lens from an *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ mouse demonstrating normal morphology.

B. Cornea, iris and lens from an *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mouse. Cornea is infiltrated by mononuclear cells and granulocytes are present in the iris and anterior chamber.

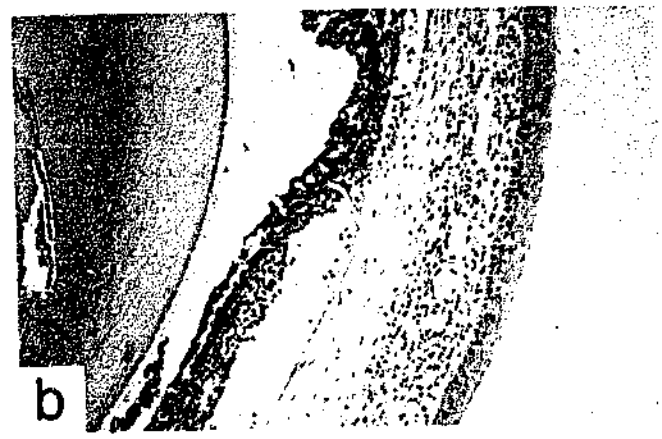
C. Skeletal muscle from an *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ mouse demonstrating normal morphology.

D. Skeletal muscle from an *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mouse. There is infiltration by T lymphocytes, macrophages and eosinophils with damage to muscle cells.

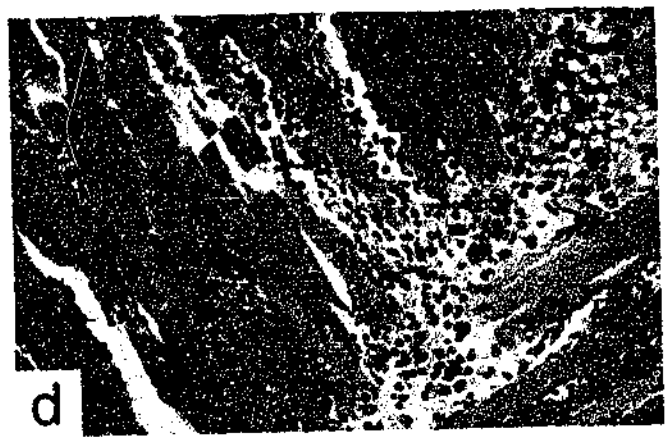
E. Lung from an *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ mouse demonstrating normal morphology.

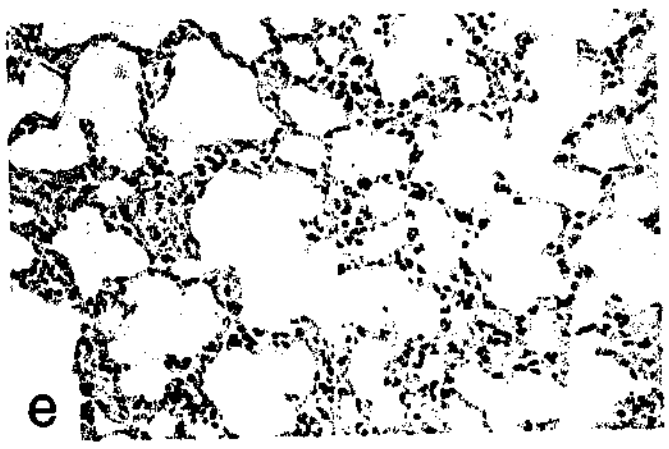
F. Lung from an *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mouse with thickening of the alveolar walls due to infiltration by T lymphocytes and macrophages.


a


b


c


d


e

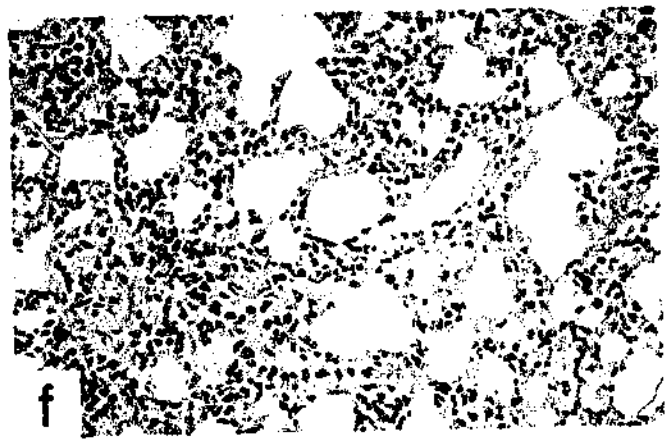
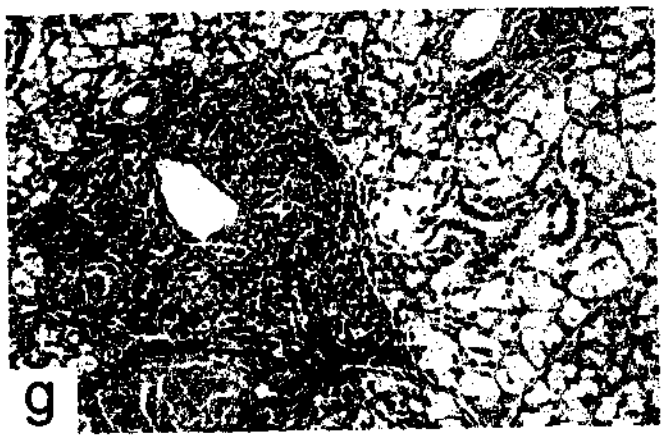

f

Figure 6.4: Pathology of *Ifnar1*$^{-/-}$ *Socs1*$^{+/+}$ Mice and *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ Mice (continued).

Mice ranging in age from 4-6 weeks of age were sacrificed and organs/tissues/bones were harvested and fixed in 10% formalin. Sections were stained with haematoxylin and eosin.

G. Salivary gland from an *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mouse showing a focal infiltrate of lymphoid cells.

H. Bone marrow from an *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mouse showing an excess proportion of neutrophilic granulocytes at various stages of maturation.


g


h

signaling could negatively impact on IFNγ responses by reducing the lethal effects of IFNγ. However, it can be concluded that IFNα/β is contributing to the neonatal effects of IFNγ. However, it can be concluded that IFNα/β is contributing to the neonatal death of the *Socs1*$^{-/-}$ mice albeit not to the same extent as IFNγ, as the removal of *Ifnar1* results in a partial rescue of the *Socs1*$^{-/-}$ mice.

6.2.4 *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ Matings Generate Genotypes in non-Mendelian Frequencies

The phenotypes of both the *Ifnar1*$^{-/-}$ (Müller *et al.*, 1994; Hwang *et al.*, 1995) and the *Ifnar2*$^{-/-}$ (Hertzog, unpublished) mice were slightly different, which suggested that IFN signaling could be effected differently by each of the receptor chains. Therefore in order to further study the effects that SOCS1 was having in the type I IFN system and to compare to the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice, *Socs1*$^{+/-}$ mice were mated to *Ifnar2*$^{+/-}$ mice to generate *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ mice. These double heterozygous mice were then interbred in conventional housing to generate mice that were null for both *Ifnar2* and *Socs1*. From these breedings there could result 9 possible genotypes that were observed however in non-Mendelian frequencies based on statistical analysis using a χ^2 test (P<0.05) most likely due to low numbers of *Ifnar2*$^{+/-}$ *Socs1*$^{-/-}$ (Table 6.3) (refer to Appendix F for χ^2 tables). This result however was most likely due to the low numbers of mice thus far generated and genotyped.

The survivals of each of the 9 possible genotypes were graphed against time in weeks post birth. Results demonstrate that the life spans of the *Socs1*$^{-/-}$ mice were not effected by the status of the *Ifnar2* allele (Figure 6.5). This figure demonstrates that heterozygote alleles for both or either one of *Ifnar2* or *Socs1* had no effect on the survival of mice exhibiting 89-100% survival. This study demonstrated at 26 weeks of age that the *Ifnar2*$^{-/-}$ mice had an 88% survival rate, which was not significantly different to the 100% survival of the wild-type mice. Mice that lacked one copy of the *Socs1* allele in the *Ifnar2*$^{-/-}$ *Socs1*$^{+/-}$ mice

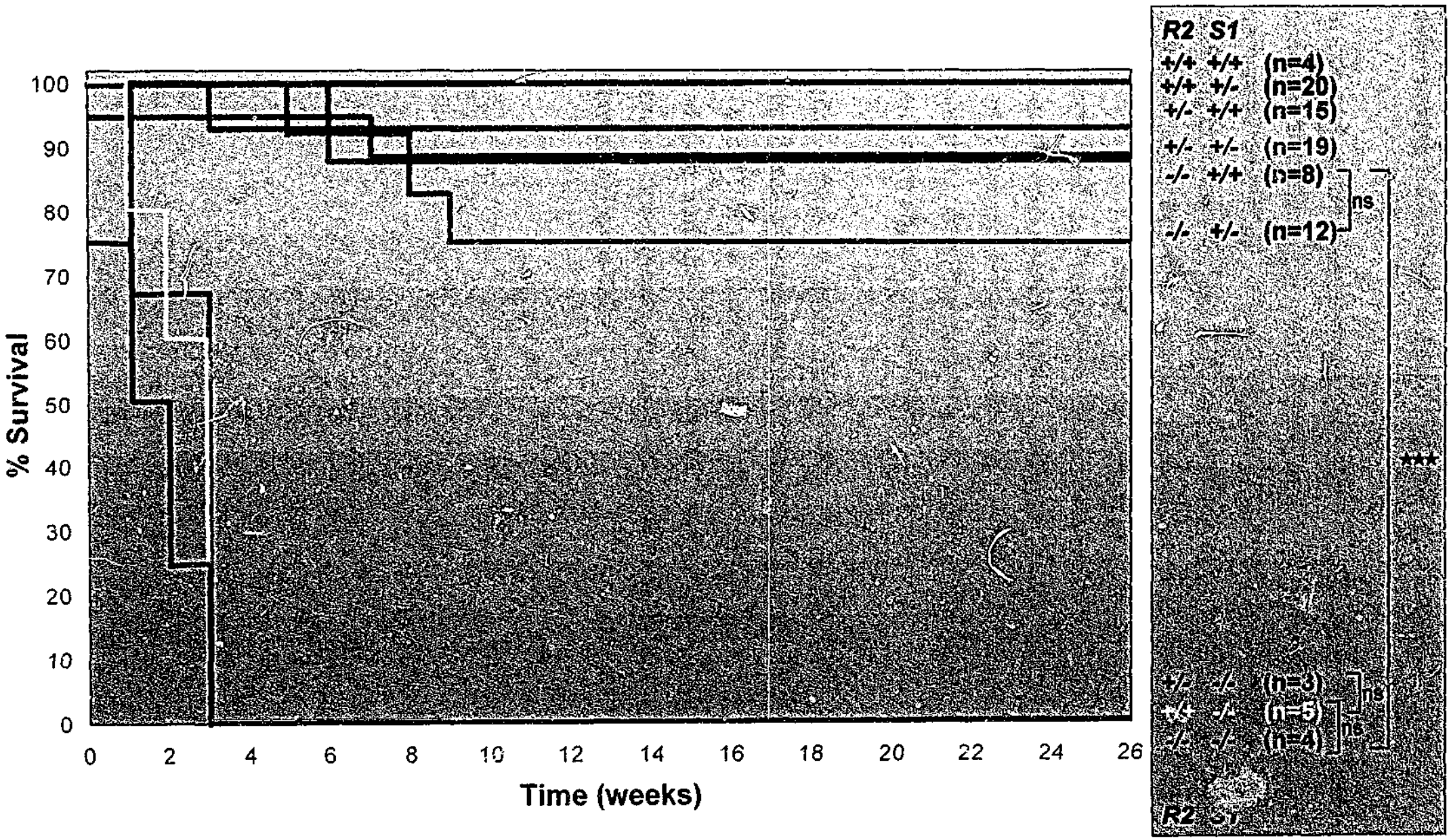
Table 6.3: Frequency of Expected Genotypes from *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ Matings.

From double heterozygous matings, each of the 9 possible genotypes were observed, however not in Mendelian ratios as determined by a χ^2 test (χ^2=17.3>P=0.05, df=8) (P<0.05).

		Number of mice from *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ crosses	
Ifnar2	***Socs1***	**Expected numbers**	**Observed numbers**
+/+	+/+	6	4
+/+	+/-	11	20
+/+	-/-	6	5
+/-	+/+	11	15
+/-	+/-	22	19
+/-	-/-	11	3
-/-	+/+	6	8
-/-	+/-	11	12
-/-	-/-	6	4
TOTALS:		90	90

Figure 6.5: Survival of Mice Generated from *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ Crosses.

From *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ X *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ breeding, each of the 9 possible genotypes were observed. Heterozygote alleles for both or either one of *Ifnar2* and *Socs1* had no effect on the survival of mice housed under conventional conditions. *Socs1*$^{-/-}$ mice suffer neonatal death by 3 weeks due to a hypersensitivity to IFNγ. *Ifnar2*$^{+/-}$ *Socs1*$^{-/-}$ —— mice did not survive past 3 weeks of age. *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$—— mice also died within 3 weeks of age. All statistics were determined by Logrank tests (*** $P<0.001$).


100
90
80
70
60
50
40
30
20
10
0
% Survival
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (weeks)
R2 S1
+/+ +/+ (n=4)
+/+ +/- (n=20)
+/- +/+ (n=15)
+/- +/- (n=19)
-/- +/+ (n=8)
-/- +/- (n=12)
ns
***
+/- -/- (n=3)
+/+ -/- (n=5)
-/- -/- (n=4)
ns
ns
R2 S1

exhibited a 74% survival rate by 26 weeks of age, which was not significantly different from the wild-type or *Ifnar2*$^{-/-}$ *Socs1*$^{+/+}$ survival rates.

In contrast to the lack of *Ifnar1* prolonging the life span of the *Socs1*$^{-/-}$, it appeared that the lack of *Ifnar2* had no effect. Results from these breedings demonstrated that at 1 week of age 80% (4/5) of the *Ifnar2*$^{+/+}$ *Socs1*$^{-/-}$ were surviving compared to 50% (2/4) of the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice. By 2 weeks of age 60% (3/5) of the *Ifnar2*$^{+/+}$ *Socs1*$^{-/-}$ were still surviving compared to 25% (1/4) of the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice. Then by 3 weeks, 100% (5/5) of *Ifnar2*$^{+/+}$ *Socs1*$^{-/-}$ mice were dead as expected, as were 100% (4/4) of *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice. These results demonstrated that in contrast to *Ifnar1*, the status of the *Ifnar2* gene is of no consequence to the survival of the *Socs1*$^{-/-}$ mouse and vice versa. This highlights a difference in how each of the receptor subunits of the type I IFN receptor are regulated by SOCS1.

The *Socs1*$^{-/-}$ mice begin to display stunted growth from 10 days of age compared to their wild-type littermates (Starr *et al.*, 1998). As the survival of the *Ifnar2*$^{+/-}$ *Socs1*$^{-/-}$ or the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ did not differ from the *Socs1*$^{-/-}$, it was expected that both the *Ifnar2*$^{+/-}$ *Socs1*$^{-/-}$ and *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ would also demonstrate stunted growth observed with smaller body size compared to wild-type littermates (Figure 6.6).

Figure 6.6: *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ Mice are Smaller in Size than their Wild-type Littermates.

A. This litter of pups generated from an *Ifnar2*$^{+/-}$ *Socs1*$^{+/-}$ matings has two pups (white and brown) that from genotyping results are both lacking *Socs1*, however only the smaller white pup is lacking *Ifnar2* as well.

B. The smaller of these two littermates is an *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ while the other littermate is a wild-type.

A.

B.

6.3 DISCUSSION

The generation of both the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice demonstrated by decreasing type I IFN signaling efficiency by removing *Ifnar1* from the *Socs1*$^{-/-}$ mice, prolonged their survival. However by 14 weeks of age all *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice had died. This demonstrated the importance of SOCS1 in mediating physiological effects of type I IFNs *in vivo*. In contrast removing *Ifnar2* from the *Socs1*$^{-/-}$ mice had no effect on their 10 to 21 day life span (Starr *et al.*, 1998; Naka *et al.*, 1998). These findings support the hypothesis that both the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ models possess different signaling capabilities.

It is interesting that one cytokine (IFNγ) can cause the dramatic neonatal lethality observed in the *Socs1*$^{-/-}$ mice. This was demonstrated by the 'rescue' of the phenotype by crossing with the *Ifng*$^{-/-}$ to generate the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice (Alexander *et al.*, 1999). However, later in life these mice suffer from inflammatory conditions including skin ulceration, pneumonia, polycystic kidneys and haematopoietic infiltration of the gut (Metcalf *et al.*, 2002). This supported that SOCS1 was an important regulator of multiple pathways and responses other than those induced by IFNγ. To further elucidate the role that SOCS1 was playing in type I IFN signaling, both *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ and *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice were generated.

Mice null for both *Ifnar1* and *Socs1* survive approximately 5-14 weeks of age. The histopathology of these mice demonstrated that they suffer from a disease characterised by infiltration of skeletal muscle and the alveolar walls in the lung by T lymphocytes and macrophages as well as infiltration of the cornea resulting in the formation of ulcers. This disease pathology is unlike the pathology seen in the *Socs1*$^{-/-}$ mice caused by a hypersensitivity to increased levels of IFNγ (Alexander *et al.*, 1999; Marine *et al.*, 1999), however is similar to the *Socs1*$^{-/-}$ *Ifng*$^{+/-}$ pathology which also includes severe myocarditis (Metcalf *et al.*, 2000). It is interesting that when the gene dosage of IFNγ is halved in the *Socs1*$^{-/-}$ mice, the pathology is remarkably similar to that of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice. This suggests that IFNγ and IFNα/β could act in a synergistic manner, together generating an increased amount of IFN signaling which is diminished

when either *Ifnar1* or one copy of *Ifng* is removed. Therefore the longer life span of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice than the *Socs1*$^{-/-}$ mice could be due to a decreased IFNγ signal resulting directly from the lack of IFNAR1. In light of previous research into the overlap between the type I and II IFN signaling pathways and biological activities, this result is consistent with the observation that IFNα/β will prime IFNγ signaling and IFNγ will prime IFNα/β signaling resulting in augmented responses in both signaling pathways (Wong *et al.*, 1998; Matsumoto *et al.*, 1999; Takaoka *et al.*, 2000). This would suggest that in the *Socs1*$^{-/-}$ mouse, both IFNγ and IFNα/β via IFNAR1 signaling contribute (IFNγ>IFNαβ) to the toxic effects observed in these mice resulting in neonatal lethality. The dependence on IFNAR1 for the synergistic IFN effect is consistent with studies demonstrating that IFNAR1 is required for the IFNGR to induce IFNγ signaling (Takaoka *et al.*, 2000). Data demonstrating that there are IFNγ independent related pathologies included polycystic kidneys, pneumonia, chronic skin ulcers and granulomas present in various organs that result from older *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice support IFNγ is not the sole cytokine responsible for disease pathologies in the *Socs1*$^{-/-}$ mice (Metcalf *et al.*, 2002).

The *Ifnar2*$^{-/-}$ mice as discussed, present with a different phenotype to the *Ifnar1*$^{-/-}$ mice (Hertzog, unpublished). As SOCS1 has a regulatory role functioning as a negative switch for IFN signaling, the effects of SOCS1 on the *Ifnar2*$^{-/-}$ mice were examined by the generation of the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice. Surprisingly the natural life spans of these mice were identical to those of the *Socs1*$^{-/-}$ mice, lending support to the difference between the signaling capabilities of the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice. The lack of effect of removing *Socs1* from *Ifnar2*$^{-/-}$ mice implies that SOCS1 is not binding to the remaining IFNAR1 chain or its associated TYK2. Due to the lack of difference in survival and gross appearance of the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ compared with the *Socs1*$^{-/-}$, there was no reason to suspect a different pathology in the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice from the *Socs1*$^{-/-}$ mouse.

The results from this chapter have demonstrated two important concepts in type I IFN signaling. Firstly, the lack of *Ifnar1* in *Socs1*$^{-/-}$ mice suggests that based

on the similar disease pathologies as *Socs1*$^{-/-}$ *Ifng*$^{+/-}$ mice, that the type I IFNs play a role in the lethality of the *Socs1*$^{-/-}$ mice. Secondly, the removal of *Socs1* from both the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice have added further evidence that there are differences in the signaling potential of both receptor chains of the type I IFN receptor complex. These differences will be the subject of the following chapter.

CHAPTER 7

Differential Signaling through IFNAR Subunits

7.1 INTRODUCTION

The preceding studies of SOCS1 regulation of type I IFN responses culminated in the conclusion that its suppressive effects were mediated by association through one chain of the receptor, IFNAR2. This emphasized previous indications of studies in the lab that there were subtle differences in the phenotypes of the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ lines (Müller *et al.*, 1994; Hwang *et al.*, 1995; Hertzog, unpublished). The assumption had originally been made that the lack of one of the receptor chains of the type I IFN receptor complex would render cells unable to respond to type I IFN. The initial immunophenotyping data which demonstrated differences in myeloid cell populations in *Ifnar1*$^{-/-}$ and thymus size as well as $CD8^+$ cells in the *Ifnar2*$^{-/-}$ as discussed in the previous chapter (Hwang *et al.*, 1995; Hertzog, unpublished), laid the foundation for the hypothesis of multiple signaling pathways emanating from the type I IFN receptor. This hypothesis was further supported firstly by the generation of the *Ifnar1*$^{-/-}$ *Ifnar2*$^{-/-}$ mice proving to be unattainable due to this genotype being fetal lethal before day 12 of gestation (Hertzog, unpublished), and secondly, by removing *Socs1*, a difference was observed in the mortality of the *Ifnar1*$^{-/-}$ compared to the *Ifnar2*$^{-/-}$ (Chapter 6). Taken together these results implied that neither of the single receptor chain knockout mice were true null mutants for type I IFN signaling and that there was residual IFNAR chain specific signaling occurring.

The fetal lethality of the *Ifnar1*$^{-/-}$ *Ifnar2*$^{-/-}$ mouse demonstrated that type I IFN was required for embryonic development and that the role in which IFN played in this process was necessary for survival. This meant that in order for the *Ifnar1*$^{-/-}$ and the *Ifnar2*$^{-/-}$ mice to survive, some residual sufficient type I IFN signaling had to be occurring. In order to search for evidence of these differences, experiments were conducted that examined the levels of the type I IFN induced protein 2'-5' OAS in the *Ifnar1*$^{-/-}$ (Hwang *et al.*, 1995). However, these experiments demonstrated that 2'-5' OAS protein was induced in wild-type mice but not in the *Ifnar1*$^{-/-}$ model after IFN treatment, suggesting that the original assumption of removing one functional receptor would result in no signaling was correct. In this chapter, expression levels of known ISGs *Mx1*,

Mhcl, *Irf1* and *Socs1* were examined before and after IFNα treatment in lung and thymus from wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice in order to elucidate any differences in signaling capabilities between the two receptor knockout models.

Microarray technology has become a prominent scientific procedure that can aid in the elucidation of global changes in gene expression or to identify evidence of unexpected signals. Therefore this was an ideal technology to search for changes in gene expression to confirm that some chain-specific signals are transduced in the *Ifnar*$^{-/-}$ mice. This technique has been utilised in order to elucidate gene expression changes in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ mice in response to IFNα4. Homogenous cell populations are beneficial in microarray experiments to minimize any artifacts due to different cell populations within experimental samples, thus reducing the signal to noise ratio. As one of the phenotypic differences between the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ was in myeloid cells, bone marrow derived macrophages, which are known to be highly sensitive to IFNα (Hwang *et al.*, 1995; Hamilton *et al.*, 1996) were used. The bone marrow aspirates could be cultured to obtain homogenous populations of bone marrow-derived macrophages as a source of substantial amounts of RNA to perform microarray analysis. Therefore, microarray techniques were employed to screen for subsets of ISGs that were differentially induced by IFNα4 via IFNAR1 or IFNAR2. By elucidating subsets of genes that were differentially expressed will lead to further understanding of the IFN signaling system.

7.2 RESULTS

7.2.1 IFN Regulation of Candidate ISGs Induced in *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ Mice

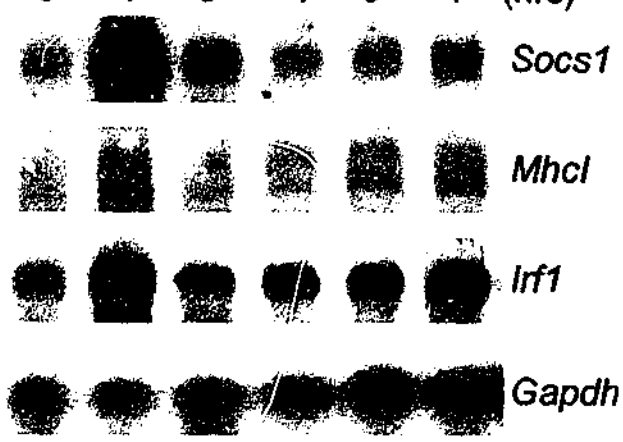
There are numerous well-characterised IFN stimulated genes with well-characterised expression patterns. Three such genes that have been utilised in these experiments are *Socs1*, *Mhcl* and *Irf1*. The DNA fragments used as Northern blot probes for these experiments were generated as outlined in section 2.2.1.

Three mice of each genotype, wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$, were either treated or untreated with IFNα4 (3.3 x10^3 IU/g) for 4 hours. Poly (A+) RNA was extracted from the lungs and the thymus (refer to 2.3.1) and subsequently transferred onto membranes that were hybridised with ^{32}P-labeled DNA fragments for *Socs1*, *Mhcl*, *Irf1* and *Gapdh* as a loading control. While there was induction of the ISGs in the wild-type, no apparent signaling could be seen from the northern blots in the lung or the thymus of the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$, with the exception of the lack of *Socs1* induction in the wild-type thymus (Figure 7.1). Based on the involvement of *Socs1* in T cell homeostasis in the thymus (Marine *et al.*, 1999; Cornish *et al.*, 2003), the lack of induction in the wild-type is of no surprise especially as there were high basal levels in the wild-type, which is consistent with the literature. When the induction levels were normalised to *Gapdh* and expressed as fold induction *Socs1*, *Mhcl* and *Irf1* were induced in the wild-type lungs 10.8, 2.6 and 4.2-fold respectively and 1.9, 2.5 and 1.6-fold respectively in the wild-type thymi (Figure 7.2). The extent of induction of ISGs 4 hours after IFNα4 treatment in both the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ lungs and thymi were not significantly different from their respective untreated controls (Figure 7.2). This suggested that the signaling cascades involved in inducing these genes were not activated in mice lacking either receptor chain.

Figure 7.1: Induction of ISGs in Wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ Mice.

Northern blots from lung and thymus taken from wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice which were injected with IFNα4 for 4 hours. Northern blot filters of the lung **A.** and thymus **B.** were sequentially hybridised with *Socs1*, *Mhcl*, *Irf1* and *Gapdh* for normalisation of mRNA levels.

A.


Lung
+/+
Ifnar1-/-
Ifnar2-/-
0 4 0 4 0 4 (hrs)
Socs1
Mhcl
Irf1
Gapdh

B.

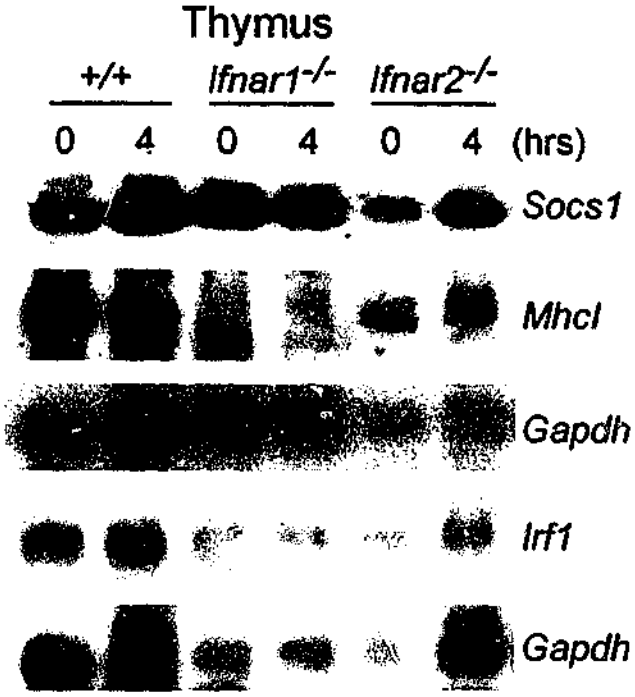

Thymus
+/+
Ifnar1-/-
Ifnar2-/-
0 4 0 4 0 4 (hrs)
Socs1
Mhcl
Gapdh
Irf1
Gapdh

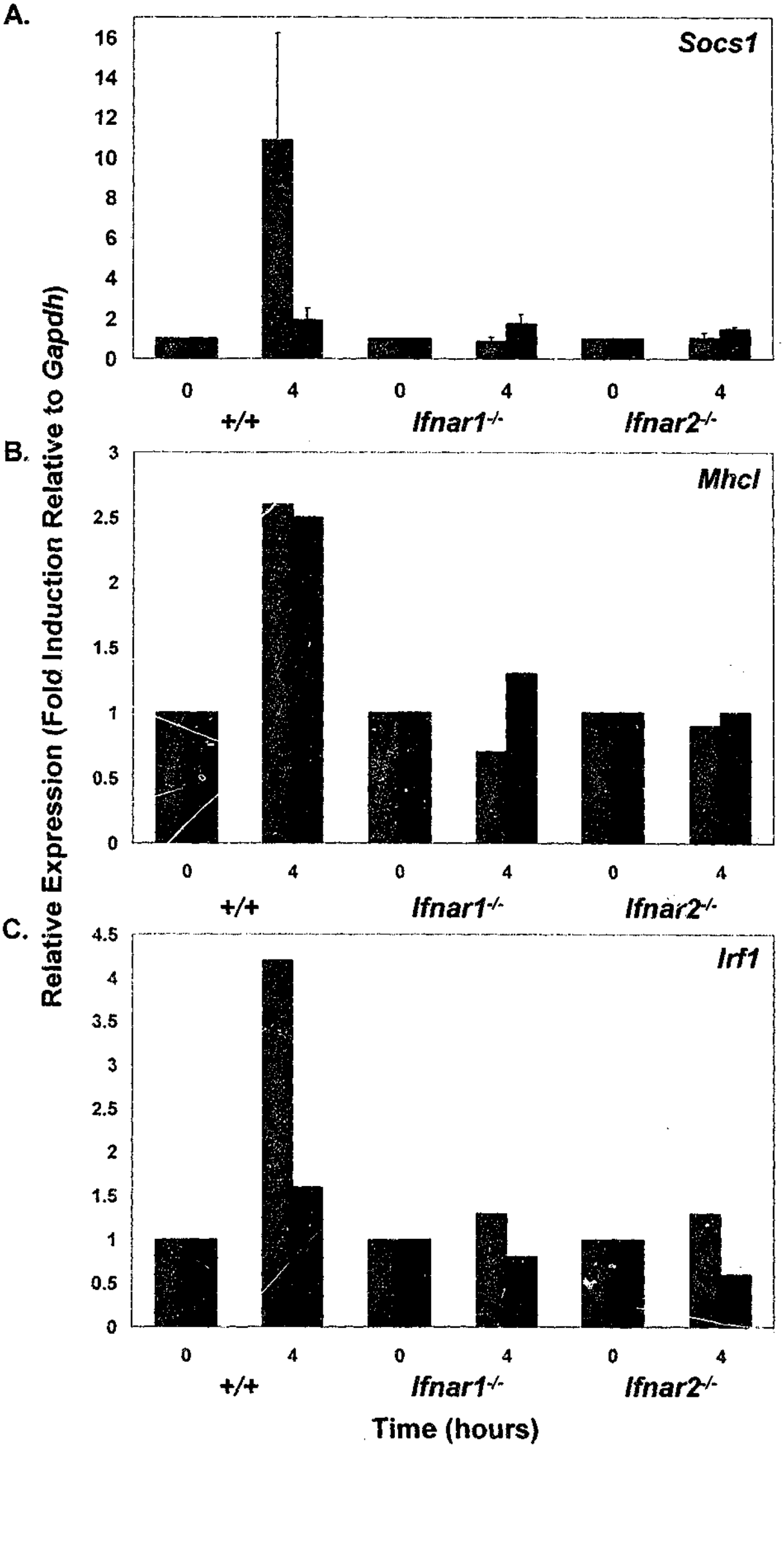
Figure 7.2: Induction of ISGs in Wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ Mice.

Poly (A)+ RNA was extracted from lungs and thymi from wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice that were injected with IFNα4. Fours hours post treatment the lungs and thymus were dissected out along with control organs that had not been treated. Subsequently mRNA was bound to Hybond C extra nitrocellulose membranes following electrophoresis. The filters were sequentially hybridised with *Socs1*, *Mhcl*, *Irf1* and *Gapdh* for normalisation of mRNA levels.

A. *Socs1* induction levels after 4 hours of IFNα4 treatment were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. In the lungs ■, a 10.8-fold induction was observed in the wild-type, however no induction was observed in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$. In the thymus ■, a marginal 1.9-fold induction was observed in wild-type, however no induction was observed in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$.

B. *Mhc1* induction levels after 4 hours of IFNα4 treatment were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. In the lungs ■, a 2.6-fold induction was observed in the wild-type, however no induction was observed in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$. In the thymus ■, a 2.5-fold induction was observed in wild-type, however no induction was observed in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$.

C. *Irf1* induction levels after 4 hours of IFNα4 treatment were normalised to *Gapdh* levels and expressed graphically as fold induction relative to time 0. In the lungs ■, a 4.2-fold induction was observed in the wild-type, however no induction was observed in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$. In the thymus ■, *Irf1* was not induced in wild-type, *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ 4 hours after treatment.


A.
Socs1
16
14
12
10
8
6
4
2
0
0
4
0
4
0
4
+/+
Ifnar1-/-
Ifnar2-/-
B.
Mhcl
3
2.5
2
1.5
1
0.5
0
0
4
0
4
0
4
+/+
Ifnar1-/-
Ifnar2-/-
C.
Irf1
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
4
0
4
0
4
+/+
Ifnar1-/-
Ifnar2-/-
Relative Expression (Fold Induction Relative to Gapdh)
Time (hours)

7.2.2 Gene Profiling of IFN Induced Genes in *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ Mice by Microarray Analysis

Both the fetal lethal phenotype of the *Ifnar1*$^{-/-}$ *Ifnar2*$^{-/-}$ mice in comparison to either of the single knockout mice, together with the different physiologies in the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice would suggest that there are ISGs that are regulated differently from each of the receptors. To investigate this, microarray technology was employed.

One of the critical components of a microarray experiment is the planning. The experimental design to study the signaling in these mice was called a "box design" experiment (Figure 7.3). *B*one *m*arrow *m*acrophages (BMMs) were generated from femural bone marrow aspirates from pools of 5 mice of each genotype: wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$. After cells were cultured for 8 days (refer to 2.5.2), half of the cells were treated with 1000 IU/ml of IFNα4 for 4 hours while the other half were left untreated as the 0 control time point. Total RNA was extracted from the BMMs (refer to 2.3.5.1) and subsequently labeled with Cy dyes and hybridised to NIA microarray slides (refer to section 2.3.5). Each sample was labeled three times with Cy3 and Cy5 that enabled dye swaps to occur, incurring more than 5 hybridisations to complete the "box design". This enabled a global population reference approach to be adopted. As can be seen in figure 7.3, the initial experiment involved the use of 9 microarray slides. For the purposes of this thesis, only slides 7, 8 and 9 were analysed hence discussed herein, i.e. gene expression changes elicited with IFN treatment for each genotype.

Gene profile experiments generate large data sets. The results of these experiments were analysed in two groups, those genes that were induced 4 hours after IFNα4 treatment and those that were repressed 4 hours after IFNα4 treatment using Genespring 5.0. A clear way to portray genes from more than one group that are behaving in a similar or mutually exclusive fashion is through the use of Venn diagrams. The Venn diagram depicting the analysis of the groups of genes that were induced higher than basal levels after 4 hours demonstrated that different genes were induced by IFNα4 in the two knockouts

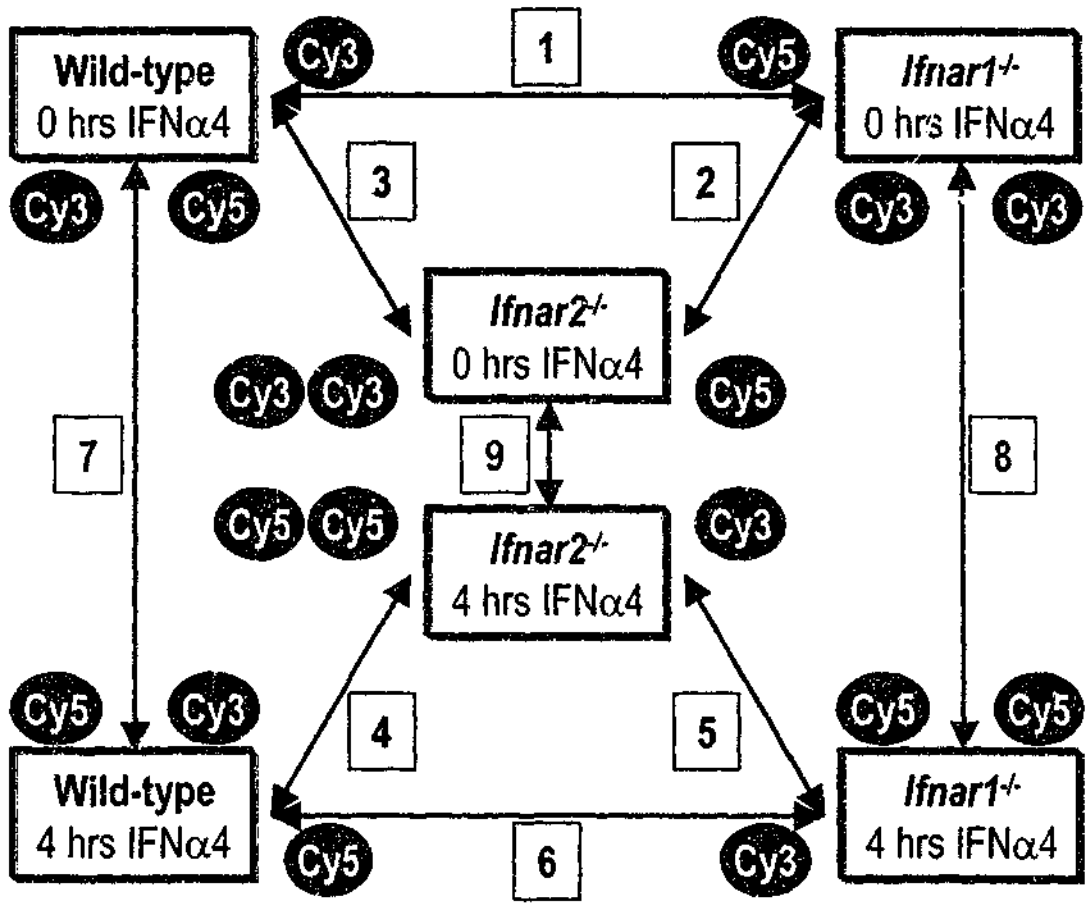


Figure 7.3: Experimental Design for a Microarray Analysis comparing BMMs from Wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ Mice.

A box experimental design was adopted to complete this experiment. The advantage of utilising a box design, 3 experiments could be put into one encompassing 9 slides [1]-[9]. Each sample was labelled three times with Cy3 and Cy5 enabling a dye swap to occur, then due to more than 5 hybridisations taking place, a global population reference approach was utilised.

(Figure 7.4). While there were 228 genes induced in the wild-type BMMs in response to IFNα4, 2 (*Riken cDNA K14* and a *murine cDNA clone*) were also induced in the *Ifnar1*$^{-/-}$. A further different 6 genes (*Mki67ip*, *Stat2*, *Wsb2-pending*, *Riken cDNA K22*, *Riken cDNA F02* and expressed sequence *C85340*) induced also in the *Ifnar2*$^{-/-}$ BMMs (Table 7.1). Interestingly this preliminary experiment produced no genes that were commonly induced in both the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ BMMs. However there were 103 out of 105 genes that were induced in *Ifnar1*$^{-/-}$ BMMs exclusively such as *methallothionein 1* and 118 out of 124 genes induced in *Ifnar2*$^{-/-}$ BMMs such as *serum/glucocorticoid regulated kinase* (refer to appendix G for extended gene lists). From this analysis there were more than 210 genes which while induced by IFNα4 in wild-type BMMs, failed to be induced in either knockout model, highlighting the absence of much of the normal IFN signaling.

Similarly for the second group of genes analysed, those repressed 4 hours after IFNα4 treatment generated a Venn diagram that again inferred the two knockout mice were able to regulate genes differently (Figure 7.5). Out of the 149 genes that were suppressed in wild-type BMMs, 3 (*Cellular nucleic acid binding protein*, *Enolase 1* and murine *cDNA clone F04*) were suppressed in the *Ifnar1*$^{-/-}$ BMMs. A further 5 different genes (*Wbscr1*, *Riken cDNA C18*, *Ube2d2*, *EST* similar to mouse ribosomal protein L34 and *H2afz*) were suppressed in BMMs derived from wild-type and *Ifnar2*$^{-/-}$ mice. The *Ifnar1*$^{-/-}$ BMMs repressed 550 genes of which 540 were exclusive to *Ifnar1*$^{-/-}$, one of which was *Dscr1*, while 7 (*Riken cDNA P05*, *Stk11*, *Snta1*, *Tmsb4x*, 2 murine cDNA clones and a gene similar to F-box and leucine rich repeat protein 5) were also repressed by *Ifnar2*$^{-/-}$ BMMs. There were 110 genes that were repressed in *Ifnar2*$^{-/-}$ derived BMMs, of which 98 were exclusive such as *Inhibitor of DNA binding 2* while 7 were shared with *Ifnar1*$^{-/-}$ BMMs and 5 shared with wild-type BMMs as already mentioned (Table 7.2) (refer to appendix G for extended gene lists).

These preliminary results have generated gene lists as expected that contain genes either induced or repressed 4 hours after IFNα4 treatment. However, interestingly these results have also generated gene lists that are exclusively

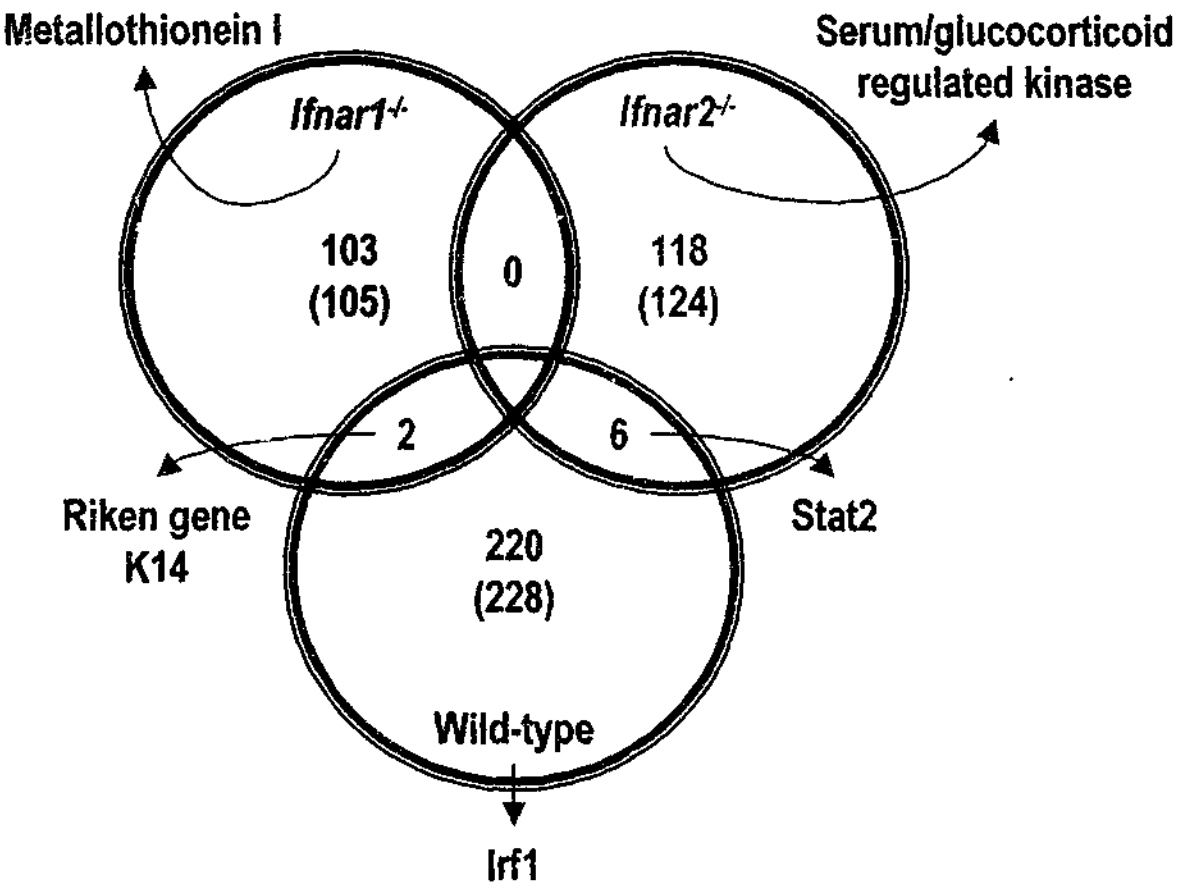


Figure 7.4: Genes which are Induced 4 Hours after IFNα4 Treatment.

Analysis of the data generated from the microarray experiment was conducted using Genespring 5.0. Expressing the data in a Venn diagram illustrates clearly that there are 2 genes that are common to the *Ifnar1*$^{-/-}$ and wild-type, 6 common between *Ifnar2*$^{-/-}$ and wild-type, while no genes on the array slide passed all the selection criteria that were common to only the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$. The genes named were selected off the lists generated to use in validation experiments.

Table 7.1: Genes that are Induced 4 Hours after IFNα4 Treatment.

This table demonstrates the genes which are common to 2 of the genotypes examined, listing the normalised ratios and the common names along with brief descriptions. The gene highlighted Stat2 was chosen for validation.

Common between *Ifnar1*$^{-/-}$ and wild-type in higher at 4 hrs vs 0 hrs				
Wild-type	***Ifnar1***$^{-/-}$	***Ifnar2***$^{-/-}$	**Common Name**	**Description**
2.7826643	2.1678066	0.82477134	2610204 K14Rik	RIKEN cDNA 2610204K14 gene
2.384574	2.2027931	1.085109		C0177B01-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0177B01 3', mRNA sequence

Common between *Ifnar2*$^{-/-}$ and wild-type in higher at 4 hrs vs 0 hrs				
Wild-type	***Ifnar1***$^{-/-}$	***Ifnar2***$^{-/-}$	**Common Name**	**Description**
2.402653	2.201452	2.821306	Mki67ip	Mki67 (FHA domain) interacting nucleolar phosphoprotein
3.792351	0.8591969	3.5641925	Stat2	Signal Transducer and Activator of Transcription 2
2.041434	0.7165008	2.0393622	Wsb2-pending	WD-40 repeat-containing protein with a SOCS box 2
2.072305	0.7064239	2.0899887	4932431F 02Rik	RIKEN cDNA 4932431F02 gene
2.195375	0.7330445	2.1326122	2610007 K22Rik	RIKEN cDNA 2610007K22 gene
3.161111	0.2764311	2.410425	C85340	expressed sequence C85340

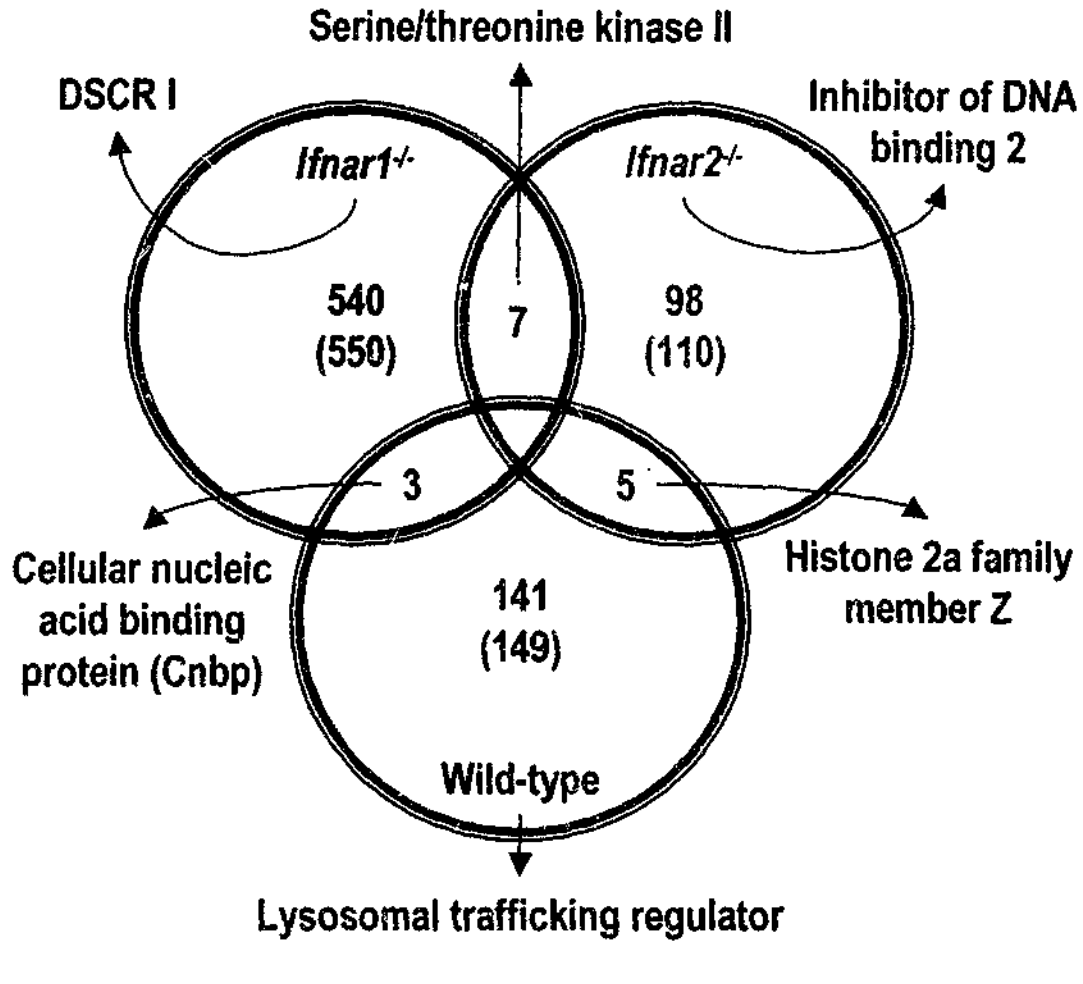


Figure 7.5: Genes that are Repressed 4 Hours after IFNα4 Treatment.

Analysis of the data generated from the microarray experiment was conducted using Genespring 5.0. Expressing the data in a Venn diagram illustrates clearly that there are 3 genes that are common to the *Ifnar1*$^{-/-}$ and wild-type, 5 common between *Ifnar2*$^{-/-}$ and wild-type, while 7 are common between only the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$. The genes named were selected off the lists generated to use in validation experiments.

Table 7.2: Genes that are Repressed 4 Hours after IFNα4 Treatment.

This table demonstrates the genes which are common to 2 of the genotypes examined, listing the normalised ratios and the common names along with brief descriptions. The gene highlighted Stk11 was chosen for validation.

Common between *Ifnar1*$^{-/-}$ and wild-type in lower at 4 hrs vs 0 hrs				
Wild-type	***Ifnar1***$^{-/-}$	***Ifnar2***$^{-/-}$	**Common Name**	**Description**
0.4271742	0.49108446	0.9109761		C0186F04-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0186F04 3', mRNA sequence
0.35285637	0.39733088	0.6051906	Cnbp	Cellular nucleic acid binding protein
0.48401332	0.33679223	0.7872604	Eno1	Enolase 1, alpha non-neuron

Common between *Ifnar2*$^{-/-}$ and wild-type in lower at 4 hrs vs 0 hrs				
Wild-type	***Ifnar1***$^{-/-}$	***Ifnar2***$^{-/-}$	**Common Name**	**Description**
0.48340875	0.6988496	0.4556848	Wbscr1	Williams-Beuren syndrome chromosome region 1 homolog (human)
0.4095217	0.7499564	0.4763838	5730591 C18Rik	RIKEN cDNA 5730591C18 gene
0.482373	0.5125914	0.3719556	Ube2d2	Ubiquitin-conjugating enzyme E2D 2
0.25507405	0.55575114	0.4151768		ESTs, Highly similar to RL34_MOUSE 60S ribosomal protein L34 [M. musculus]
0.38859394	0.90740305	0.4425301	H2afz	H2A histone family, member Z

Common between *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ in lower at 4 hrs vs 0 hrs				
Wild-type	***Ifnar1***$^{-/-}$	***Ifnar2***$^{-/-}$	**Common Name**	**Description**
0.7768876	0.4864248	0.3652894	1600025 P05Rik	RIKEN cDNA 1600025P05 gene
1.2355074	0.3094233	0.4215781		H3054E07-5 NIA Mouse 15K cDNA Clone set Mus musculus cDNA clone H3054E07 5', mRNA sequence
1.2560157	0.2894521	0.3709047		H3011F02-3 NIA Mouse 15K cDNA Clone set Mus musculus cDNA clone H3011F02 3', mRNA sequence
2.0317912	0.3037369	0.3740411	Stk11	Serine/threonine kinase 11
0.6958864	0.3036315	0.461387	Snta1	Syntrophin, acidic 1
0.6062657	0.4432785	0.4069243	Tmsb4x	Thymosin, beta 4, X chromosome
0.7204451	0.4937837	0.4847639		Mus musculus, Similar to F-box and leucine-rich repeat protein 5, clone IMAGE: 3989687, mRNA

induced/repressed by each of the *Ifnar* knockout models which lends support to the hypothesis of this study that the two *Ifnar* knockout models were capable of initiating type I IFN signaling.

7.2.2.1 Validation of Microarray Differences

Microarray analysis is a technology that enables gene expression differences to be isolated. Once isolated these genes of interest need to be validated by either biological replicates of the same microarray analysis, Northern blot analysis or real time PCR. Therefore in order to form conclusions from the data generated from the microarray analysis described, genes were selected for validation by real time PCR. The advantage of real time PCR over Northern analysis is that it is quicker and requires less starting material. From the group of genes induced 4 hours after IFNα4 treatment, 4 genes were selected for validation in each of the genotypes: *Stat2*, *Serum/glucocorticoid regulated kinase*, *Metallothionein1* and *Irf1*. Microarray analysis demonstrated *Stat2* to be induced by IFNα4 in both wild-type and *Ifnar2*$^{-/-}$ BMMs 4.8-fold and 3-fold respectively. When validated by real time PCR induction levels in wild-type and *Ifnar2*$^{-/-}$ BMMs were 3.7-fold and 3.6-fold respectively (Figure 7.6A). *Serum/glucocorticoid regulated kinase* was induced 3.3-fold in *Ifnar2*$^{-/-}$ BMMs exclusively from the microarray analysis. However these results were not validated by real time PCR, as the induction level in *Ifnar2*$^{-/-}$ BMMs was only 1.1-fold (Figure 7.6B). *Metallothionein1* was induced 4.6-fold in *Ifnar1*$^{-/-}$ BMMs exclusively according to the microarray analysis. When validated by real time PCR the induction level was 3.3-fold. However real time PCR results also demonstrated that *methallothionein1* was suppressed 5-fold in *Ifnar2*$^{-/-}$ treated BMMs which was not observed in the microarray analysis (Figure 7.6C). From the microarray analysis, *Irf1* was exclusively induced in wild-type BMMs 4.8-fold. Previously, *Irf1* was induced in the wild-type lung 4.2-fold by Northern hybridisation, while no induction was observed in either *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ (Figure 7.2).

Of the group of genes that were suppressed after treatment with IFNα4, 2 genes, *Serine/threonine kinase II* and *Dscr1* were selected for validation by real

Figure 7.6: Validation of ISGs Induced 4 Hours after IFNα4 Treatment by Real Time PCR.

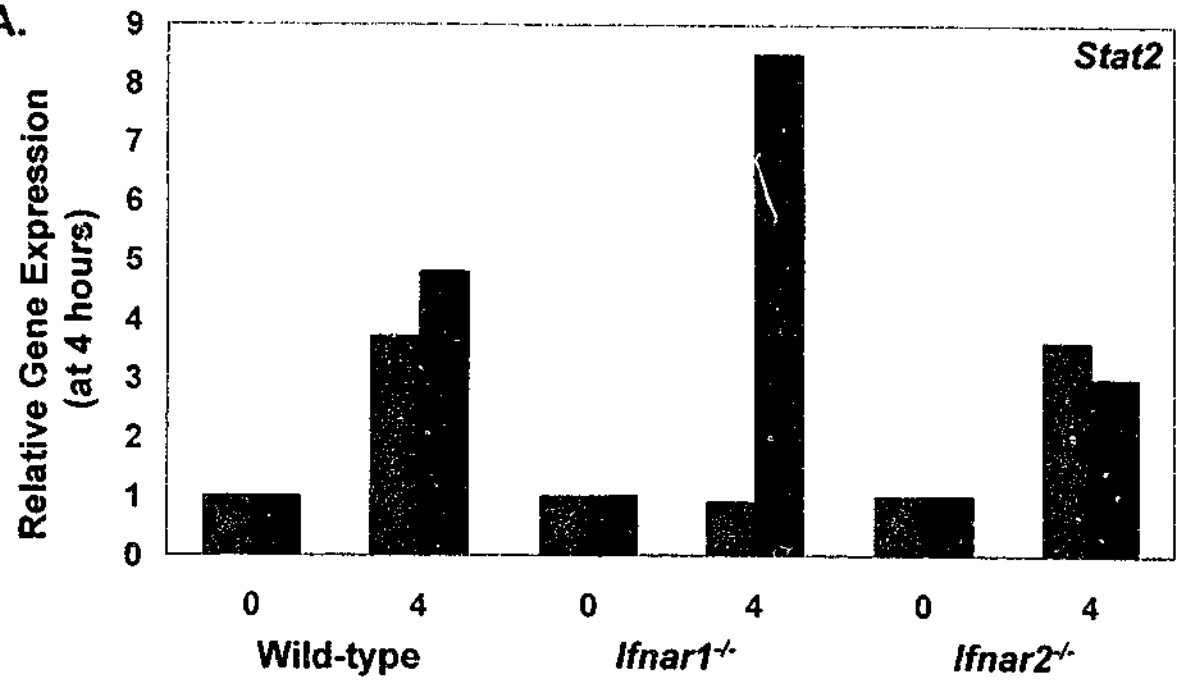
Bone marrow macrophages isolated from wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice were treated with IFNα4 for 4 hours. Total RNA was extracted and indirectly labelled with Cy3 and Cy5 for microarray hybridisation. From the results a set of genes were chosen to validate by real time PCR.

A. *Stat2* was induced 4.8-fold, 8.5-fold and 3-fold according to the microarray data ■ in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively. Validation by real time PCR ■ demonstrated *Stat2* to be induced 3.7-fold, repressed 1.2-fold and induced 3.6-fold in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively.

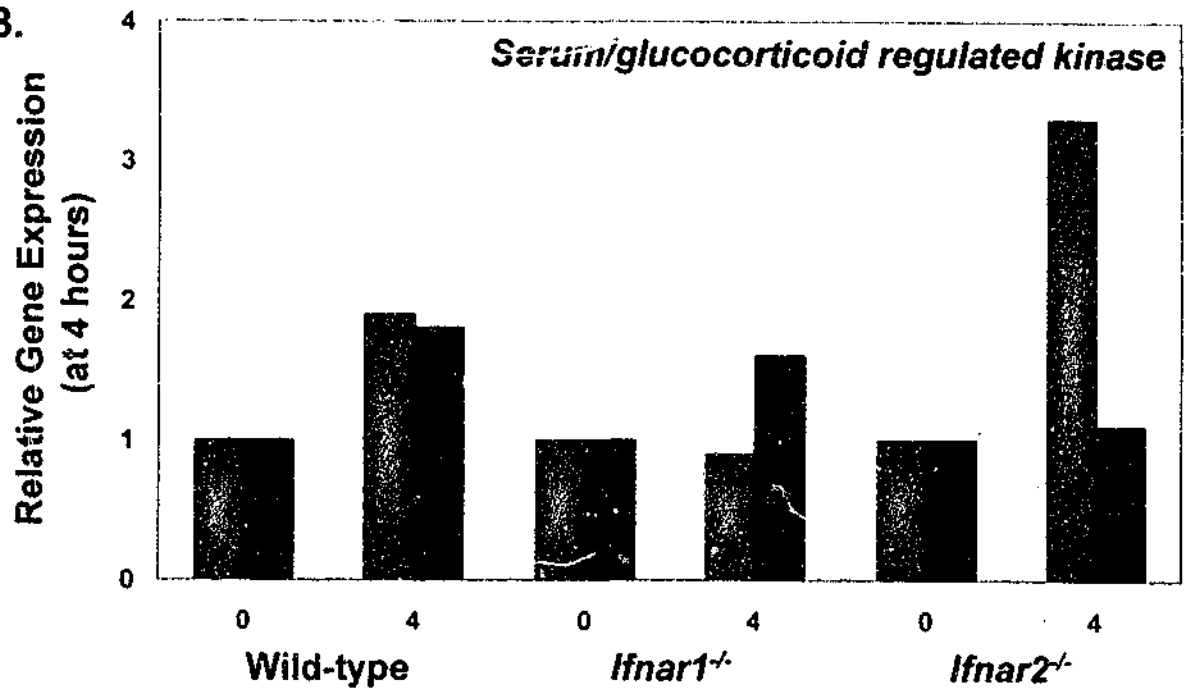
B. *Serum/glucocorticoid regulated kinase* was induced 1.9-fold, repressed 1.3-fold and induced 3.3-fold according to the microarray data ■ in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively. Validation by real time PCR ■ demonstrated *serum/glucocorticoid regulated kinase* was not induced with fold induction levels measuring 1.8-fold, 1.6-fold and 1.1-fold in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively.

C. *Metallothionein I* was repressed 1.1-fold, induced 4.6-fold and 1.1-fold according to the microarray data ■ in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively. Validation by real time PCR ■ demonstrated *metallothionein I* to be induced 2.05-fold, 3.3-fold and repressed 5-fold in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively.

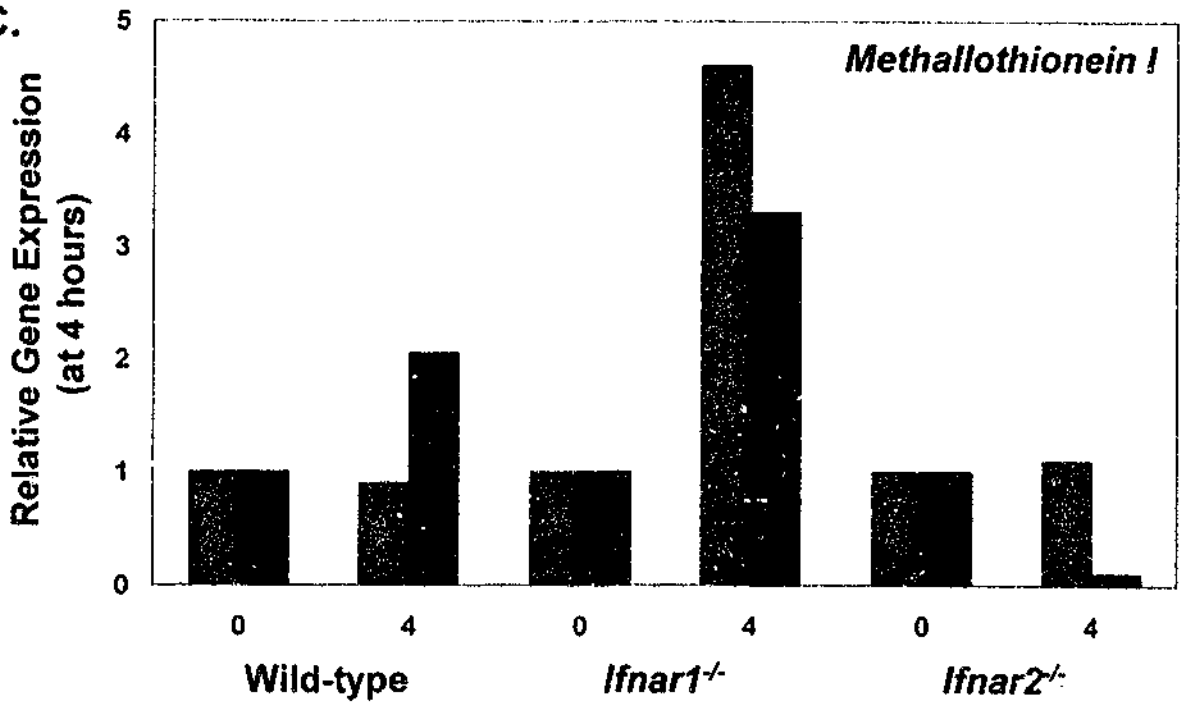
A.


Stat2
Relative Gene Expression (at 4 hours)
0
1
2
3
4
5
6
7
8
9
0
4
0
4
0
4
Wild-type
Ifnar1-/-
Ifnar2-/-

B.


Serum/glucocorticoid regulated kinase
Relative Gene Expression (at 4 hours)
0
1
2
3
4
0
4
0
4
0
4
Wild-type
Ifnar1-/-
Ifnar2-/-

C.


Methallothionein I
Relative Gene Expression (at 4 hours)
0
1
2
3
4
5
0
4
0
4
0
4
Wild-type
Ifnar1-/-
Ifnar2-/-

time PCR. From the microarray analysis, *serine/threonine kinase II* was induced in both *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ BMMs by IFNα4 3.3-fold and 2.5-fold respectively. When validated by real time PCR the induction levels in *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ BMMs were 5-fold and 8-fold respectively (Figure 7.7A). *Dscr1*, from the microarray analysis was repressed 2.5-fold in *Ifnar1*$^{-/-}$ IFNα4 treated BMMs exclusively. However real time PCR results demonstrated that *Dscr1* was repressed 1.2-fold in *Ifnar1*$^{-/-}$ treated BMMs (Figure 7.7B). Even though a few of these genes could not be validated to support the array data, the importance of gene validation has been highlighted.

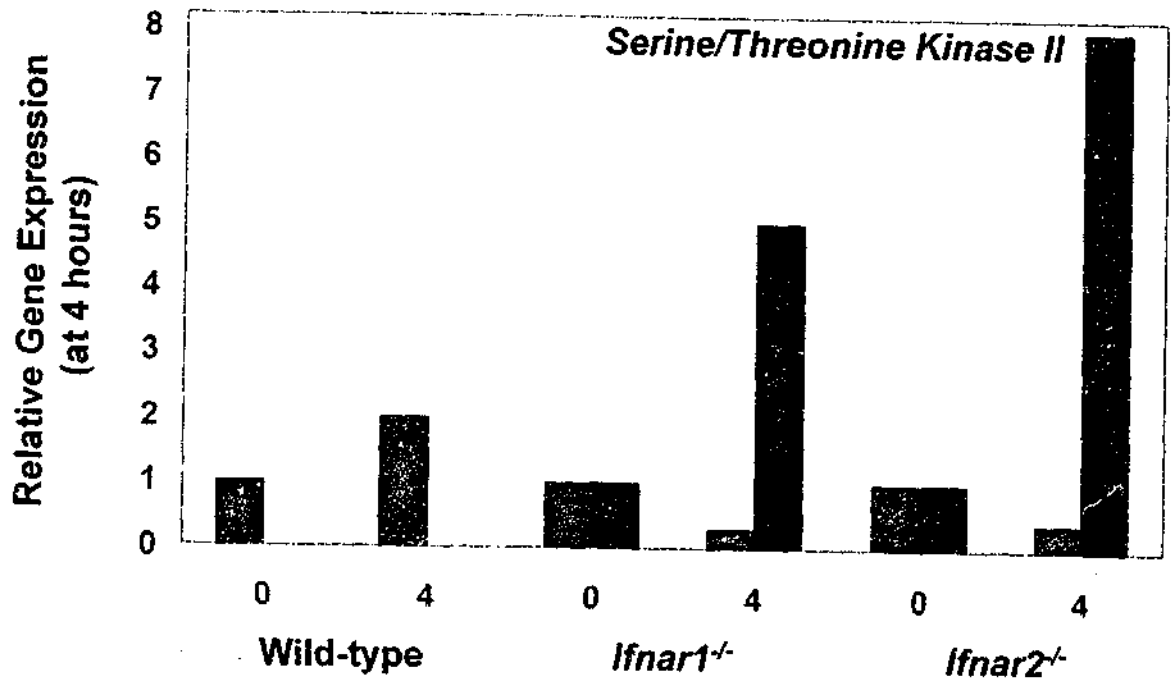
Figure 7.7: Validation of ISGs Repressed 4 Hours after IFNα4 Treatment by Real Time PCR.

Bone marrow macrophages isolated from wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice and subsequently treated with IFNα4 for 4 hours. Total RNA was extracted and indirectly labelled with Cy3 and Cy5 for microarray hybridisation. From the results a set of genes were chosen to validate by real time PCR.

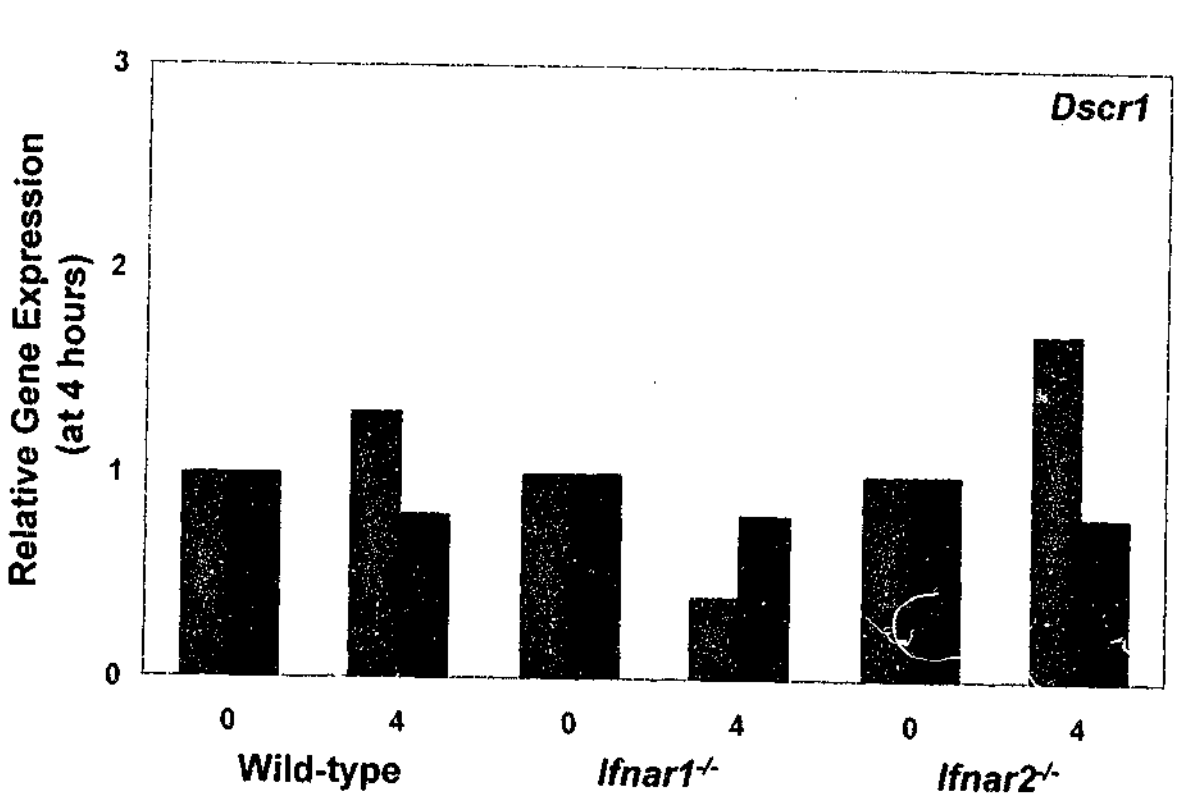
A. *Serine/threonine kinase II* was induced 2-fold, repressed 3.3-fold and 2.5-fold according to the microarray data ■ in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively. Validation by real time PCR ■ demonstrated *serine/threonine kinase II* to be induced 5-fold and 8-fold in the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively.

B. *Dscr1* was induced 1.3-fold up, repressed 2.5-fold and induced 1.7-fold according to the microarray data ■ in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively. According to real time PCR validations ■ , *Dscr1* was neither induced or repressed 4 hours after IFNα4 treatment with fold repressions of 1.3-fold, 1.2-fold and 1.2-fold in the wild-type, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells respectively.

A.


Serine/Threonine Kinase II
Relative Gene Expression (at 4 hours)
8
7
6
5
4
3
2
1
0
0
4
0
4
0
4
Wild-type
Ifnar1-/-
Ifnar2-/-

B.


Dscr1
Relative Gene Expression (at 4 hours)
3
2
1
0
0
4
0
4
0
4
Wild-type
Ifnar1-/-
Ifnar2-/-

7.3 DISCUSSION

Deciphering groups of genes that are regulated specifically by one of the IFNAR subunits and not the other has the potential to further elucidate the more specific roles of each component of the type I IFN receptor. Northern blot data on the induction of specific ISGs, *Socs1*, *Mhcl* and *Irf1* have demonstrated that there are common signaling pathways that require both IFNAR1 and IFNAR2 and associated signaling components in order to transduce a signal. Array results also confirmed this with *Cib1* (calcium and integrin binding 1), *Tgtp* (T cell specific GTPase), *Irf1* and *Jak2* to mention a few that were induced in the wild-type but not in either knockout (refer to Appendix G). The induction data of *Socs1* from this study indicates that the signaling pathways resulting in the expression of certain ISGs are also tissue/organ specific. *Socs1* was induced in wild-type lungs however not in the thymus, perhaps related to the high basal level in that organ.

Microarray technology is a way to look for global changes in different models under different conditions. It was utilised in this project in order to isolate genes that were induced in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ exclusively. Heterogeneity of the cell populations in organs can result in changes in gene expression in minor cell populations being masked. Therefore homogenous cell types are preferential. To overcome the heterogeneity problem microarray analysis was carried out using bone marrow derived macrophages that were cultured for 8 days to generate a homogenous cell population.

Microarray results have demonstrated that the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice are able to control the expression of different sets of genes, both those that were induced or repressed 4 hours post IFNα4 treatment. This is the first molecular evidence supporting the hypothesis that there are signals that require only one subunit of the IFNAR unit. Of the genes that were induced, 4 known genes and 1 unknown were selected for validation. Of these, three produced similar gene expression changes as the array experiment. *Irf1* a well-characterised ISG was induced in wild-type BMMs but not in either the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ BMMs implying that the signaling pathway utilised required a fully associated type I IFN

receptor unit similar to *Cib*, *Tgtp* and *Jak2* as above. This result was consistent with previous Northern analysis in the lung however induction in the thymus was not evident, which again demonstrates organ specificity with *Irf1* gene induction. *Stat2*, an integral component of ISGF3, was induced according to microarray and real time PCR results in both the wild-type and *Ifnar2*$^{-/-}$ BMMs to similar intensities, but not in the *Ifnar1*$^{-/-}$ BMMs. The third gene, *methallothionein I* was chosen as microarray data suggested that it was a gene that was induced in only the *Ifnar1*$^{-/-}$. However experiments vaildating this gene demonstrated that *methallothionein I* was induced in the wild-type but interestingly was not in the *Ifnar2*$^{-/-}$. Therefore, *methallothionein I* was still a gene of interest as validation experiments demonstrate that this gene is an IFNAR2 subunit specific gene, meaning that the presence of IFNAR2 was sufficient to induce the expression of this gene by IFNα4.

From the genes that could be validated from the array experiment, it was clear that there are groups of genes that behave differently in response to IFNα4. How they respond is dependent on which of the IFNAR subunits are present. Proof of concept was achieved to support the original hypothesis that signaling pathways emanate from the individual subunits by virtue of different sets of genes being regulated in the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice four hours after IFNα4 treatment.

The discovery of IFNAR chain-specific groups of ISGs could lead to a better understanding of how type I IFNs signal. The fact that different genes are induced by the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ cells infer that different factors are utilised in the signaling cascade initiated by each of the chains, otherwise no induction of ISGs in a chain-specific manner would be expected. There are many factors known to associate with the IFNAR chains for signaling to occur. These include TYK2, JAK1, CD45, STAT1, STAT2 and STAT3 to mention a few. Some of these are known to associate with particular chains (e.g.: TYK2 and IFNAR1 (Richter *et al.*, 1998), JAK1 with IFNAR2 (Stark & Kerr, 1992; Kotenko *et al.*, 1999), STAT2 with IFNAR2 (Nadeau *et al.*, 1999)). Until now it has been assumed that both chains with their associated kinases were necessary for all

signaling. This data implies that one chain and its associated signaling factors can elicit some signal that has a biological outcome. Therefore it would be interesting to characterise IFNAR-specific signaling pathways. This might ultimately enable us to understand the molecular basis for the pleiotrophic effects of IFN. This could form the basis for the development of drugs that target by agonist or antagonist chain-specific biological effects.

From experiments carried out in this chapter, it can be seen that the removal of one receptor chain can result in genes being regulated in a different fashion to wild-type conditions. This was evident as there were a large proportion of genes that were induced/repressed in the *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$, but not in the wild-type. The differences observed may merely be artifacts of removing a gene that would not naturally occur. But this does demonstrate that in wild-type cells the presence of each chain (and its associated signaling components) suppresses the signaling potential of the other chain. One example of this is the association of SOCS1 with IFNAR2. This was shown by infection experiments of both *Ifnar1*$^{-/-}$ and *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice that suggested that SOCS1 was able to regulate IFNAR2 function, as the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ mice were able to sustain a longer infection (Chapter 4). While there is no negative regulation demonstrating association with IFNAR1, there are several candidates (refer to Chapter 1) such as other negative regulators SHP1 and CD45 (Haque *et al.*, 1998; Irie-Sasaki *et al.*, 2001). Further elucidation of the interactions of the negative regulatory systems with the *Ifnar*$^{-/-}$ mice will aide in our understanding of how the duration and nature of signals is regulated.

Characterisation of the IFNAR chain-specific regulated genes might identify genes or gene sets that have known functions or disease associations. Such data could provide evidence for testing particular disease models for example in the *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ to identify disease relevant signals. In the long term this approach could also form the basis for new therapeutic targeting strategies for particular diseases.

CHAPTER 8

General Discussion

8.1 GENERAL DISCUSSION

The type I IFNs are a group of secreted cellular proteins that elicit diverse effects in numerous cell types by binding to and activating a common cell surface receptor, IFNAR. Initiated signaling pathways induce proteins that function in antiviral, antiproliferative and immunoregulatory activities (Pestka *et al.*, 1987). As a result of these pleiotrophic activities IFNs are thought to play important roles in the regulation of physiological and pathological processes.

Receptor knockout studies have been useful in understanding type I IFN signaling. Microarray results demonstrated in BMMs cultured from both *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice that signaling does occur in the absence of either receptor resulting in gene transcript expression (Chapter 7). This was the first molecular evidence supporting the hypothesis that residual signaling was emanating from each of the single receptor chains, which was formed based on the different phenotypes observed in the *Ifnar1*$^{-/-}$ (Müller *et al.*, 1994; Hwang *et al.*, 1995) and *Ifnar2*$^{-/-}$ mice (Hertzog, unpublished). The fetal lethality of the double *Ifnar1*$^{-/-}$ *Ifnar2*$^{-/-}$ added further support to this hypothesis (Hertzog, unpublished). Additional experimentation including dosage and time courses and validation is required to confirm the observed gene changes and to decipher what role they are playing *in vivo*. Bioinformatic tools such as gene clustering could group genes based on their similarity in expression profile and functional characterisation. For example they could be grouped based on whether the gene expression is increased or decreased, or expressed in one genotype only or in a combination of two and not one. The genes could be coregulated over a timecourse or promoter analysis could be used to look for common transcription factor elements such as STAT-driven genes, AP1 or NFκB to mention a few. The genes may also be grouped based on gene ontology classification based on function, subcellular localisation or domain structure. Potentially, this could then identify groups of genes with similar functions that are induced/repressed by one chain and not the other, which may account for the elicitation of specific physiological and pathological effects of IFNs. One interesting area to pursue would be to identify the signal transduction mechanisms responsible for these changes in gene expression (Chapter 7).

An intriguing implication of the IFNAR-specific signaling is that it demonstrates that IFN can bind to a partly functional receptor in the absence of *Ifnar1* or *Ifnar2*. This is contrary to current models, but very important to characterise. One possibility is that IFN engages a single chain which then multimerises with other signaling factors to induce signal transduction. An alternative is that IFN engages one chain and an unknown receptor or accessory molecule is recruited to generate a signal. These will be important areas to study and IFNAR-specific ISG's identified here could provide specific readouts for signaling from these complexes.

Another interesting feature of the IFNAR chain-specific gene sets is that many genes are not induced in the wild-type. This suggests that each IFNAR chain (and its associated factors) has a suppressive effect on signaling in wild-type cells. It will be important to characterise the nature and mechanism of these suppressors. One candidate, which is a major focus of this thesis is SOCS1.

Due to the pleiotrophic effects of IFNs, a tight regulatory system would be advantageous to gain the beneficial effects of IFN without the harmful side effects including toxicity which is a major problem with current IFN based therapies (Gutterman *et al.*, 1994). In addition, it has been demonstrated in this thesis that a system of negative regulation exists of which the SOCS proteins play a key role. These proteins, particularly SOCS1, can be induced by a variety of cytokines including IL6, IL4, growth hormone, prolactin and thrombopoietin to mention a few (Yoshimura *et al.*, 1995; Starr *et al.*, 1997; Masuhara *et al.*, 1997; Naka *et al.*, 1997; Adams *et al.*, 1998; Bjorbaek *et al.*, 1998; Helman *et al.*, 1998; Sakamoto *et al.*, 1998; Suzuki *et al.*, 1998). *In vitro* studies (Chapter 3) demonstrated that both type I and II IFNs could be added to the list of *Socs1* inducing cytokines. Interestingly, type I IFN induced *Socs1* expression maximally and had then decreased before IFNγ induced *Socs1* transcripts were detectable (Chapter 3). This raised the question, is IFNγ induced *Socs1* an indirect effect. In the sense of regulation, what makes the SOCS proteins interesting, is their ability to inhibit the effects of their inducing cytokines (Nicholson & Hilton, 1998; Yoshimura, 1998; Alexander, 2002). In

particular, both SOCS1 and SOCS3 were shown to inhibit the antiviral effects elicited by IFNs in cell cultures (Chapter 3; Song & Shuai, 1998).

The generation of the *Socs1*$^{-/-}$ mouse model demonstrated the importance of SOCS1 function *in vivo* (Starr *et al.*, 1998; Naka *et al.*, 1998). These mice suffer from neonatal lethality due to a hypersensitivity to IFNγ, which resulted from an increase in IFNγ production and action (Starr *et al.*, 1998; Metcalf *et al.*, 1999; Alexander *et al.*, 1999; Marine *et al.*, 1999). Therefore it was concluded that SOCS1 plays a crucial role in balancing the lethal toxic effects of IFNγ with the beneficial effects by inhibiting IFNγ induced signaling (Alexander *et al.*, 1999). Since SOCS1 *in vitro* was induced by and regulated not only IFNγ but also IFNα and IFNβ, the important question was whether SOCS1 also regulated type I IFN responses *in vivo*.

The characteristic features of type I IFNs is their rapid induction by virus and their potent antiviral actions. The *Socs1*$^{-/-}$ mice were found to be more resistant to virus as they were able to endure a SFV infection for a prolonged period of time compared to their wild-type littermate controls (Alexander *et al.*, 1999; Chapter 4). This could be due to increased IFN production as was found for IFNγ or an increase in response to type I IFN. When IFN levels were measured from the serum of *Socs1*$^{-/-}$ mice 48 hours after infection, they were lower than their respective wild-type controls. This suggested that the increased viral resistance was unlikely resultant of increased IFN production and highlighted a difference between virus induced IFN (likely to be type I) and endogenous IFNγ levels in *Socs1*$^{-/-}$ mice. The viral titres measured in various organs from both *Socs1*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ mice were consistently lower (100-1000-fold) than in the same organs from respective wild-type control mice. Therefore demonstrating a direct effect on viral replication, a hallmark of type I IFN action (Pestka *et al.*, 1987).

The data presented in this thesis proves that SOCS1-mediated antiviral effects were via type I and not type II signaling for a number of reasons. These were the increased viral resistance observed in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$, the resistance

observed in the *Ifng*$^{-/-}$ was not different to the wild-type, using an anti-IFNα/β antibody decreased the resistance observed in the *Socs1*$^{-/-}$ *Ifng*$^{-/-}$ and finally the increased resistance to infection in the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ compared to the *Ifnar1*$^{-/-}$. The generation of *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ as well as *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mouse models enabled further elucidation of the effects *Socs1* had on IFN responses. As discussed above, removing *Socs1* from *Ifnar1*$^{-/-}$ mice improved their resistance to SFV infection. This suggested that SOCS1 was regulating type I IFN antiviral activity through the IFNAR2 subunit.

These two double knockout mouse models also added further support to the difference between each of the single *Ifnar*$^{-/-}$ models (Müller *et al.*, 1994; Hwang *et al.*, 1995; Hertzog, unpublished) with the *Ifnar2*$^{-/-}$ *Socs1*$^{-/-}$ mice showing no difference in mortality compared with *Socs1*$^{-/-}$ mice, whereas the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ did (Chapter 6). This suggested that SOCS1 was effecting each of the receptor chains differently.

Ifnar1$^{-/-}$ *Socs1*$^{-/-}$ mice are characterised by infiltrates of the lungs, skeletal muscle and cornea often causing the formation of ulcers. However suprisingly, the liver was not effected as in the *Socs1*$^{-/-}$ (Starr *et al.*, 1998; Metcalf *et al.*, 1999), but rather these mice resembled the phenotype of the *Socs1*$^{-/-}$ *Ifng*$^{+/-}$ (Metcalf *et al.*, 2000). This similarity in phenotype suggests an overlap in function of IFNα/β and IFNγ. The type I IFNs could have even contributed to the disease in the *Socs1*$^{-/-}$ mice, however went undetected due to the severity of the disease elicited due to a hypersensitivity to IFNγ. It is known that IFNγ can induce components of the type I IFN signaling system and likewise the type I IFNs can induce IFNγ signaling components (Wong *et al.*, 1998; Matsumoto *et al.*, 1999; Takaoka *et al.*, 2000). The similarities in the pathology of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ and *Socs1*$^{-/-}$ *Ifng*$^{+/-}$ suggest a possible synergistic effect between the two IFN types *in vivo*. The longer life span of the *Ifnar1*$^{-/-}$ *Socs1*$^{-/-}$ compared to the *Socs1*$^{-/-}$ could be due to a decreased IFNγ signal resulting from the lack of the IFNAR1 subunit of the type I IFN receptor (Takaoka *et al.*, 2000). Thus, the data in this thesis is consistent with the suggestion that IFNAR1 acts as an adaptor between the type I and II IFN signaling pathways where IFNα/β primes

for IFNγ responses (Takaoka *et al.*, 2000). Indeed work from this laboratory has demonstrated that IFNγ responses are diminished in *Ifnar*$^{-/-}$ mice (Hertzog, personal communication). This could be measured and further characterised directly by measuring IFNγ inducible genes/proteins in the presence and absence of IFNAR1 alone and in combination with *Socs1* removal.

Thus SOCS1 has a profound effect on acute virus infection via type I IFN mediated signals. Perhaps drugs to amplify acute antiviral responses therapeutically could directly target SOCS1. However clinical use of IFN in many cases involves chronic administration resulting in positive therapeutic outcomes, but with side effects including nausea, neutropenia, fever, headaches and even autoimmune disease. A major challenge in this field is whether these effects of IFN could be separated, i.e. increase the therapeutic benefits and decrease the side effects. The panel of genetically modified mice generated in this work could be useful in this regard. Since the combination of *Ifnar1*$^{-/-}$ *and Ifnar2*$^{-/-}$ mice with the *Socs1*$^{-/-}$ mice generate specific signals, chronic injection of IFN and measurements of responses typical of clinical side effects (white blood cell counts, fever and autoimmune indications) might identify whether these effects are preferentially elicited via one chain or modified by one specific regulator such as SOCS1.

APPENDICES

APPENDIX A
Buffers and Solutions

Buffer A (2'-5' OAS Assay)

1 M Glycine, pH 2.0.

Buffer C (2'-5' OAS)

10 mM Hepes, 5 mM $MgCl_2$, 50 mM KCl, 5 mM Dithiothreitol, 20% (v/v) Glycerol, pH 7.5.

5.7 M Cesium Chloride Solution

5.7 M CsCl, 0.83% (v/v) 3 M NaOAc (pH 6.0).

100 x Denhardts Solution

2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) Polyvinylpirollidone (PVP).

DNA Loading Dye

0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol, 1.5% (w/v) Ficoll type 400.

KALB Lysis Buffer

1 mM EDTA, 150 mM NaCl, 50 mM Tris (pH 7.5), 1% (v/v) Triton X, pH 7.4.

Luria-Bertani (LB) Agar

1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 1% (w/v) NaCl, 1.5% (w/v) Agar.

LB Broth

1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 1% (w/v) NaCl, pH 7.0.

Lysis Buffer (2'-5' OAS Assay)

20 mM Hepes, 5 mM $MgCl_2$, 120 mM KCl, 5 mM Dithiothreitol, 10% (v/v) Glycerol, 0.5% (v/v) NP-40, pH 7.5.

10 x MOPS

4.186% (w/v) MOPS, 10 mM EDTA, 50 mM NaOAc, pH 7.0.

Northern Hybridisation Solution

50% (v/v) Deionised formamide, 1% (w/v) SDS, 1 M NaCl, 10% (w/v) Dextran sulphate, 100 µg/ml Denatured herring sperm DNA.

Northern Pre-hybridisation Solution

50% (v/v) Deionised formamide, 5 x SSC, 1 x Denhardts solution, 100 µg/ml Denatured herring sperm DNA.

10 x PBS

0.8% (w/v) NaCl, 0.145% (w/v) Na_2HPO_4, 0.02% (w/v) KCl, 0.02% (w/v) KH_2PO_4, pH 7.4.

Piero's Binding Buffer

5 M Guanidine Thiocynate, 10 mM Tris (pH 7.0), 0.03% Gelatin, 2 ng/µl yeast tRNA.

Reaction Buffer

10 mM Hepes, 5 mM $MgCl_2$, 5 mM Dithiothreitol, 10 mM Creatine phosphate, 10% (v/v) Glycerol, 2.5 mM ATP, 3 mg/ml Creatine kinase, 40 µg/ml Poly rI : Poly rC, pH 7.5.

Reaction Mixture

50 µl ^{32}P α ATP per ml of Reaction buffer.

RNA Binding Buffer

0.1% (v/v) SDS, 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0).

RNA Elution Buffer

0.1% (v/v) SDS, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0).

RNA Extraction Buffer

0.5% (v/v) SDS, 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0).

RNA Loading Dye

0.2% (w/v) Bromophenol blue, 0.2% (w/v) Xylene cyanol, 20% (w/v) Ficoll type 400.

RNA Suspension Buffer (RSB)

1 x MOPS, 50% (v/v) Formamide, 6.23% (v/v) Formaldehyde.

RNA Wash Buffer

0.1% (v/v) SDS, 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0).

2 x Sample Buffer

0.125 M Tris (pH 6.8), 25% (v/v) Glycerol, 2.5% (v/v) SDS, 0.0003% (w/v) Bromophenol blue, 5% (v/v) Mercaptoethanol.

10% SDS

10% (w/v) Sodium dodecyl sulfate.

1 x SDS PAGE Running Buffer

25 mM Tris-HCl, 192 mM Glycine, 0.1% (w/v) SDS.

SOC Medium

2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 0.01 M NaCl, 2.5 mM KCL. Adjust to pH 7.0. Add 10mM glucose, 10mM $MgCl_2$, 10mM $MgSO_4$ prior to use.

Solution D

4 M Guanidinium thiocyanate, 25 mM Sodium citrate (pH 7.0), 0.5% (w/v) Sodium lauryl sarkosinate in DEPC-treated water. Add β-mercaptoethanol to a final 0.1 M prior to use.

Southern Pre-Hybridisation/Hybrisation Solution

10% (w/v) Dextran sulphate, 1% (w/v) SDS, 1 M NaCl, 100 µg/ml single stranded sonicated Herring sperm DNA.

Southern Transfer Buffer

1.6% (w/v) NaOH

Stock Solution

10% (w/v) Diatomaceous Earth, 3.5 M Guanidine HCl, 0.1 mM EDTA, 200 mM NaOAc (pH 4.8-5.0).

20 x SSC

17.5% (w/v) NaCl, 8.8% (w/v) Sodium citrate, pH 7.0.

1 x TAE

40 mM Tris-acetate, 1 mM EDTA, pH 8.0.

Tail Lysis Buffer

1% (v/v) SDS, 0.1 M NaCl, 0.1 M EDTA, 0.05 M Tris-HCl, pH 8.0.

1 x TE Buffer

10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Terrific Broth (TB)

1.2% (w/v) Bacto-tryptone, 2.4% (w/v) Bacto-yeast extract, 0.4% (v/v) Glycerol. Add 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 prior to use.

0.25% Trypsin/EDTA

0.25% (w/v) Trypsin, 1 mM EDTA.

Western Transfer Buffer

1 x SDS PAGE Running buffer, 20% (v/v) Methanol.

APPENDIX B
List of Suppliers

Amersham Pharmacia	Buckinghamshire, UK
Bartell Instruments	Heidelberg West, VIC, Australia
BDH Biochemicals/Merck	Poole, UK
Beckman Instruments	Fullerton, CA, USA
Becton Dickinson	Lincoln Park, NJ, USA
BioRad	Hercules, CA, USA
Canberra Packard	Australia
Crown Scientific	Burwood, VIC, Australia
CSL	Parkville, VIC, Australia
DAKO	Carpinteria, CA, USA
Dynavac	Melbourne, VIC, Australia
Genetic MicroSystems	Woburn, MA, USA
Gibco-BRL (Life Technologies)	Paisley, UK
Heraeus Instruments	Hanau, Germany
Hoefer	San Fransico, CA, USA
Invitrogen	Mount Waverly, VIC, Australia
Kodak Scientific Imaging Film	Rochester, NY, USA
Millipore Corporation	Bedford, MA, USA
NEN Life Science Products	Boronia, VIC, Australia
Perkin Elmer	Norwalk, Connecticut, USA
Pierce	Rockford, IL, USA
Proligo	Lismore, NSW, Australia
Promega	Madison, WI, USA
Qiagen	Chatsworth, CA, USA
Roche Diagnostics	Mannheim, Germany
Sigma Chemical Company	Saint Louis, MO, USA
Stratagene	La Jolla, CA, USA

APPENDIX C
List of Equipment

Agarose gel electrophoresis	Mini-sub Cell GT, BioRad
β-scintillation counter	1900TR, Canberra Packard
Centrifuges (large)	J2-21 M/E, Beckman
	Biofuge stratos, Heraeus Instruments
Cell Incubator	HERA Cell, Heraeus Instruments
Homogeniser	Ultra Turrax T25, Crown Scientific
Hybridisation ovens	XTRON H2002, Bartell Instruments
Microarray Scanner	GMS 418 Array Scanner, Genetic Microsystems
Microcentrifuge	1-15 Sigma, Quantum Scientific
LightCycler	Roche Diagnostic
PCR Machines	GeneAmp PCR System 2400, Applied Biosystems
Phosphorimager	FLA-2000, Fujifilm
Protein gel caster	Mighty Small multiple gel caster SE200, Hoefer Scientific Instruments, Amersham Pharmacia
SDS PAGE electrophoresis	Mighty Small II SE250, Hoefer Scientific apparatus Instruments, Amersham Pharmacia
Spectrophotometer	Lambda Bio20, Perkin Elmer
UV illuminator	Gel Doc 1000, BioRad
Ultracentrifuge	TL-100 and L8-70M, Beckman
Vacuum Oven	Dynavac
Western transfer apparatus	TE77 SemiPhor, Hoefer Scientific Instruments, Amersham Pharmacia

APPENDIX D
Oligonucleotides

Oligonucleotide	Sequence (5'-3')	Orientation
Cellular nucleic acid binding protein (Cnbp) F	TGC ACC AAG GTG AAG TGC TA	Sense
Cellular nucleic acid binding protein (Cnbp) R	TTG GCC AGT GAA GAG GAT TC	Antisense
Down Syndrome chromosome region I (DSCR1) F	CAT CTT GGG AAG CCA GGA AA	Sense
Down Syndrome chromosome region I (DSCR1) R	TGC TGC TCT CCC ACA GGT TA	Antisense
Gapdh F	GTC ATA CCA GGA AAT CAG C	Sense
Gapdh R	CTG CCA CCC AGA AGA CTG TGG	Antisense
H2A histone family, member Z F	TTG GTT GGT TGG AAG GCT AA	Sense
H2A histone family, member Z R	ACA CAT CCA CAA ATC GCT GA	Antisense
Ifnar2 F	GCA GGA AGT ATG CCT AGC GAG G	Sense
Ifnar2 R	AGA GAA CAA GTC TGG CCC ACC C	Antisense
Inhibitor of DNA binding 2 F	CAG CAT TCA GTA GGC TCG TG	Sense
Inhibitor of DNA binding 2 R	GCC TGG ACT GTG ATA CCG TT	Antisense
Irf1 F	GCT CTA GGG CCA GTG CTA TG	Sense
Irf1 R	CTG AGG TGT AAG GCA GAG GC	Antisense
Lysosomal trafficking regulator F	ACG AAA GAC CAT TCA CCA GG	Sense
Lysosomal trafficking regulator R	CAC ATG GAC CAA ATG TCT GC	Antisense
Metallothionein 1 F	TCC TGC AAG AAG AGC TGC TG	Sense
Metallothionein 1 R	TAG GAA GAC GCT GGG TTG GT	Antisense
Riken gene K14 F	CTG GCT GCA AGA CAA CAG AC	Sense
Riken gene K14 R	CTG GTT GTG GCC ATA CTG TG	Antisense
Serine/Threonine kinase 11 F	TCG ACT CCA CCG AGG TAA TC	Sense
Serine/Threonine kinase 11 R	CTT CTT GAC GTT GGC CTC TC	Antisense
Serum/glucocorticoid regulated kinase F	CAT AGC ACA CTC ACG CCA CT	Sense
Serum/glucocorticoid regulated kinase R	AGG TCT AAG AGG AAT CCC CG	Antisense
Stat2 F	GTC TGC CTC CTT GGT TCT TG	Sense
Stat2 R	AGA ACA GTC CAG GAA GGG GT	Antisense

APPENDIX E
Sources of Plasmid DNA Containing ISG cDNA

The ISG cDNAs used in this study were received from the following laboratories:

Cis	Dr Douglas Hilton, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
D3	Dr Ricky Johnstone, The Austin Research Institute, Melbourne, Australia
Irf1	Dr Harada, Nippon Boehringer Ingelheim, Hyogo, Japan
Mhcl	Dr Bernadette Scott, Monash Institute of Reproduction, Melbourne, Australia
Mx1	Dr Peter Staeheli, Institute of Immunology and Virology, University of Zürich, Switzerland
Socs1	Dr Douglas Hilton, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
Socs2	Dr Douglas Hilton, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
Socs3	Dr Douglas Hilton, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

APPENDIX F
χ^2 Tests

Frequency of Genotypes from Ifnar1$^{+/-}$ Socs1$^{+/-}$ Matings.

Before the redistribution of the additional 10 pups that were recorded, however not genotyped as they died before 21 days.

Genotype *Ifnar1 Socs1*	Number Observed	Number Expected	Deviation (Obs-Exp)	Deviation^2	Deviation^2 / Expected
+/+ +/+	19	14	5	25	1.786
+/+ +/-	31	27	4	16	0.593
+/+ -/-	8	14	-6	36	2.571
+/- +/+	30	27	3	9	0.333
+/- +/-	51	55	-4	16	0.291
+/- -/-	12	27	-15	225	8.333
-/- +/+	11	14	-3	9	0.643
-/- +/-	35	27	8	64	2.370
-/- -/-	12	14	-2	4	0.286
					χ^2=17.203

Degrees of freedom = 8

To accept that the genotypes are distributed in a Medelian frequency χ^2<15.51

χ^2=17.2, therefore a non-Mendelian frequency is observed (P<0.05).

After the redistribution of the additional 10 pups that were recorded, however not genotyped as they died before 21 days.

Genotype *Ifnar1 Socs1*	Number Observed	Number Expected	Deviation (Obs-Exp)	Deviation^2	Deviation^2 / Expected
+/+ +/+	19	14	5	25	1.786
+/+ +/-	31	27	4	16	0.593
+/+ -/-	11.3	14	-2.7	7.29	0.521
+/- +/+	30	27	3	9	0.333
+/- +/-	51	55	-4	16	0.291
+/- -/-	18.6	27	-8.4	70.56	2.613
-/- +/+	11	14	-3	9	0.643
-/- +/-	35	27	8	64	2.370
-/- -/-	12	14	-2	4	0.286
					χ^2=9.436

Degrees of freedom = 8

To accept that the genotypes are distributed in a Medelian frequency χ^2<15.51

χ^2=9.44, therefore a Mendelian frequency is observed (P>0.05).

Frequency of Genotypes from Ifnar2$^{+/-}$ Socs1$^{+/-}$ Matings.

Genotype *Ifnar2 Socs1*	Number Observed	Number Expected	Deviation (Obs-Exp)	Deviation^2	Deviation^2 / Expected
+/+ +/+	4	6	-2	4	0.667
+/+ +/-	20	11	9	81	7.364
+/+ -/-	5	6	-1	1	0.167
+/- +/+	15	11	4	16	1.455
+/- +/-	19	22	-3	9	0.409
+/- -/-	3	11	-8	64	5.818
-/- +/+	8	6	2	4	0.667
-/- +/-	12	11	1	1	0.091
-/- -/-	4	6	-2	4	0.667
					χ^2=17.305

Degrees of freedom = 8

Tc accept that the genotypes are distributed in a Medelian frequency χ^2<15.51

χ^2=17.3, therefore a non-Mendelian frequency is observed (P<0.05).

APPENDIX G
Microarray Genelists

0 and 4 hour comparisons IFN a4 treatment (only wt higher at 0 vs 4 hrs)					
4 hours IFN a4	**wt**	**IFNAR 1 ko**	**IFNAR 2 ko**		
Systematic	*Normalized*	*Normalized*	*Normalized*	*Common*	*Description*
BG077144	0.4995535	0.789587	0.9471585	Ing1	inhibitor of growth family, member 1
BG065738	0.4983145	1.461892	0.9308487	Arhgef3	Rho guanine nucleotide exchange factor (GEF) 3
BG065732	0.496628	1.4206393	0.9123897		H3034F02-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3034F02 3', mRNA sequence.
BG070430	0.4961315	0.6004778	0.6890991	Glb1	galactosidase, beta 1
BG077479	0.4948585	1.9895202	0.7845584		ESTs
BG068831	0.4944122	0.1496849	2.0002315		ESTs, Weakly similar to RIKEN cDNA 9030605E16 [Mus musculus] [M.musculus]
BG081279	0.4923776	0.53696	0.9972007	B4galt6	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6
BG065586	0.4917581	0.5083548	1.349205		Mus musculus, clone IMAGE:4506466, mRNA
BG068763	0.4917481	0.2413067	1.4373217		ESTs
BG068675	0.4892331	0.71312	0.9405906	5033405 K12Rik	RIKEN cDNA 5033405K12 gene
BG066669	0.4890203	1.1285416	1.0137091		ESTs
AW539280	0.4871283	0.9849941	1.1673468	5330422 J23Rik	RIKEN cDNA 5330422J23 gene
BG079961	0.4864725	1.0754085	0.7217182	Ssr4	signal sequence receptor, delta
BG065756	0.4853131	1.2931275	1.0351852	Emcn-pending	Endomucin
BG082355	0.4842372	0.8037203	1.1497086	Abcd3	ATP-binding cassette, sub-family D (ALD), member 3
BG088162	0.4842084	1.0127667	1.0264335	LOC192 196	CGI-74-like SR-rich
AW549495	0.4817439	1.0548339	0.7629831		L0051E04-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0051E04 3', mRNA sequence.
BG073496	0.4796515	0.6473563	0.5991065	3110069 K09Rik	RIKEN cDNA 3110069K09 gene
BG065735	0.4794991	0.7953809	0.8566384	Clecsf8	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 8
C76805	0.4791872	1.1940655	1.0052773	Myla	myosin light chain, alkali, cardiac atria
BG064793	0.4783922	1.5539467	0.875074		
BG088280	0.4779792	0.8293992	1.9153771	Cdon	cell adhesion molecule-related/down-regulated by oncogenes
BG077071	0.4778113	0.701862	0.7658118	2310011 F05Rik	RIKEN cDNA 2310011F05 gene
BG078832	0.4772911	0.6118165	0.7332003	Wtap	Wilms' tumour 1-associating protein
BG078522	0.4765627	0.8064769	0.2445547	Sec14l2	SEC14-like 2 (S. cerevisiae)
BG064198	0.4679707	0.9094103	0.7641158	AA40845 1	expressed sequence AA408451
AU022057	0.4674901	1.3099581	0.7255732	2810011	RIKEN cDNA 2810011A17 gene

				A17Rik; Rab32; AU022057	
BG063933	0.4668604	0.4301187	0.5485216	Dpagt2	dolichyl-phosphate alpha-N-acetylglucosaminephosphotransferase 2
BG080680	0.4654671		0.8754038	1700034M03Rik	RIKEN cDNA 1700034M03 gene
BG068581	0.4654639	1.0098735	0.6058034		ESTs
BG068195	0.463557	0.378202	0.5928079	Abce1	ATP-binding cassette, sub-family E (OABP), member 1
BG085490	0.462351	1.3263768	0.741969	Tpt1	tumor protein, translationally-controlled 1
BG077218	0.4612837	0.8856924	1.1520747	1110008E19Rik	RIKEN cDNA 1110008E19 gene
BG079589	0.4607844	1.4362167	1.0782027	Ap3m1	adaptor-related protein complex AP-3, mu 1 subunit
BG081616	0.4589952	1.0333967	0.714001	Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
BG076596	0.4587116	0.8275454	0.8054837	Ubb	ubiquitin B
BG068269	0.4580292	1.5639179	0.8072008	Anxa7	annexin A7
BG087043	0.457338	0.6020834	1.1555198	Hspa8	heat shock 70kD protein 8
BG066848	0.4547269	1.321541	0.90539	AI429612	expressed sequence AI429612
BG079886	0.4544681		0.8175558	1700030C10Rik	RIKEN cDNA 1700030C10 gene
AW544560	0.4537164	1.1428255	0.7939917	Calm1	calmodulin 1
BG065834	0.4528709	1.0796623	0.8205876	C77080	expressed sequence C77080
BG068408	0.452152	0.6363728	0.7896975	AU022812	expressed sequence AU022812
BG067684	0.4479656	0.2692496	0.5937851		Mus musculus, clone IMAGE:3676222, mRNA
BG068457	0.4429605	0.279828	1.3024682		ESTs, Weakly similar to S24407 formin isoform IV - mouse [M.musculus]
BG071230	0.4420686	0.7559638	0.6164816	4933411J24Rik	RIKEN cDNA 4933411J24 gene
BG078516	0.4405971	0.5576252	0.5095813	D16Ertd454e	DNA segment, Chr 16, ERATO Doi 454, expressed
BG065111	0.435891	0.6186322	0.5336884	H2afz	H2A histone family, member Z
BG063885	0.4353858	1.2880108	0.5792207	AW146109	expressed sequence AW146109
BG066468	0.4346433	1.4219668	0.8887045		EST
BG073051	0.4311009	1.054632	0.8665377	Ptma	prothymosin alpha
BG081582	0.4306863	0.8677639	1.0185754	1110003E01Rik	RIKEN cDNA 1110003E01 gene
BG068293	0.4263649	1.4487164	0.7804755		ESTs
BG073953	0.4219498	1.8721044	0.6571537		H3129B07-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3129B07 3', mRNA sequence.
BG086313	0.4214509	0.8551093	1.2906897	Gna12	guanine nucleotide binding protein, alpha 12
BG081584	0.4213718	0.7860965	0.9986472	4432405K22Rik	RIKEN cDNA 4432405K22 gene
BG064447	0.4198743	0.3269621	0.8673106		EST
BG077249	0.4197676	0.776957	0.8790969	Ranbp9	RAN binding protein 9

C81132	0.4193326	1.1310985	1.0674089		C81132 Mouse 3.5-dpc blastocyst cDNA Mus musculus cDNA clone J0094B12 3' similar to Mus domesticus hydrophobic protein mRNA sequence.
BG071689	0.4193299	0.8775502	0.6340088	4432405 K22Rik	RIKEN cDNA 4432405K22 gene
BG077034	0.4193105	0.5066416	1.2009299	Arfrp1	ADP-ribosylation factor related protein 1
BG088213	0.417432	1.1337426	0.6100152	Rps15	ribosomal protein S15
BG065281	0.4174234	0.5996698	0.9742234	Gmfb	glia maturation factor, beta
BG066834	0.4147276	0.6300357	0.7128962	4930506 D01Rik	RIKEN cDNA 4930506D01 gene
BG080391	0.4133438	0.2431872	1.1297505	Arsdr1	androgen-regulated short-chain dehydrogenase/reductase 1
BG065902	0.4126076	1.5356737	0.9198744	D8Ertd9 1e	DNA segment, Chr 8, ERATO Doi 91, expressed
BG063844	0.4090039	0.9959735	1.4887624	Lcp1	lymphocyte cytosolic protein 1
BG079850	0.4072707	0.9018878	1.0207168	Got1	glutamate oxaloacetate transaminase 1, soluble
BG066992	0.4059309	0.3291093	1.068652	Sca10	spinocerebellar ataxia 10 homolog (human)
BG067220	0.4043289	1.4721162	0.7476656	Btg4	B-cell translocation gene 4
BG081505	0.4012961	0.2299569	14.725001	Pcm1	pericentriolar material 1
BG076932	0.4010193	1.1315556	0.9296497	Anxa1	annexin A1
BG079910	0.3997981	1.5389444	0.8973227		ESTs, Highly similar to VPP1_MOUSE Vacuolar proton translocating ATPase 116 kDa subunit A isoform 1 (Clathrin-coated vesicle/synaptic vesicle proton pump 116 kDa subunit) (Vacuolar proton pump subunit 1) (Vacuolar adenosine triphosphatase subunit Ac116) [M.musculus]
BG065589	0.3996559		0.0612328		ESTs
AW538981	0.3996368	2.620024	0.6915562		C0114C01-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0114C01 3', mRNA sequence.
BG070875	0.3977034	0.8773961	0.831151	D10Ertd 773e	DNA segment, Chr 10, ERATO Doi 773, expressed
BG068399	0.3967408	0.2590853	0.5077902	4930432 O21Rik	RIKEN cDNA 4930432O21 gene
BG068030	0.3944	0.1684235	0.7071974	AU02174 9	expressed sequence AU021749
BG072628	0.3941401	0.6175251	0.5964275	Scml3	sex comb on midleg-like 3 (Drosophila)
BG081111	0.3932637	0.8058965	0.8295081	Top1	topoisomerase (DNA) I
BG066573	0.3896809	1.0701486	0.6088589		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG085400	0.3869667	1.6710988	1.021119	Dab2	disabled homolog 2 (Drosophila)
BG063843	0.3864409	0.9452148	1.0104747	Cox7a2l	cytochrome c oxidase subunit VIIa polypeptide 2-like
AW536733	0.3847812	2.05	3.1197042	Lyst	lysosomal trafficking regulator
BG068627	0.3814709	1.0307164	0.8049887		ESTs
BG066700	0.3745786	1.1814592	0.4236433	2210008 F15Rik	RIKEN cDNA 2210008F15 gene

BG068279	0.3678676	0.3752094	0.7879632	LOC216565	KIAA0903-like
BG072696	0.367781	1.4336535	0.7736536	Tpt1	tumor protein, translationally-controlled 1
BG066474	0.3660304	1.3577982	0.8815797	C79562	expressed sequence C79562
BG080169	0.3611409	0.4618988	1.2964538	Dia1	diaphorase 1 (NADH)
BG078872	0.3587501		1.1672707	Pfkfb2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
BG068288	0.3560707	1.223261	1.1954062	Slc21a10	solute carrier family 21 (organic anion transporter), member 10
BG070676	0.3523464	0.942164	0.9548336	2600011C06Rik	RIKEN cDNA 2600011C06 gene
BG070847	0.3471901	0.1154462	1.3446473		ESTs
C77369	0.344966	0.8729076	0.6785168	2400007G07Rik; C77369; AI462345	RIKEN cDNA 2400007G07 gene
AW552551	0.3436865	0.6528716	0.5180811	AI506168	expressed sequence AI506168
BG064222	0.3424083	0.460504	1.2009578	AW545847	expressed sequence AW545847
BG080248	0.3415474	0.4747327	0.7737923	MGC12070	hypothetical protein MGC12070
BG081793	0.341201		0.6825907	BC027802	hypothetical protein BC027802
BG079512	0.3299641	0.6174158	1.045117	2310022K01Rik	RIKEN cDNA 2310022K01 gene
BG067677	0.3278933		0.4015392	D16Wsu83e	DNA segment, Chr 16, Wayne State University 83, expressed
BG068292	0.3251853	1.4230245	0.7708651	D8Ertd594e	DNA segment, Chr 8, ERATO Doi 594, expressed
BG078930	0.3104343		1.2589729	Fkbp4	FK506 binding protein 4 (59 kDa)
AU023849	0.3051285		0.7474558		ESTs
BG079360	0.3037187	0.3648539	1.3078715		ESTs
BG066434	0.2968976		0.5967228	D5Ertd236e	DNA segment, Chr 5, ERATO Doi 236, expressed
BG078524	0.2936295	1.0246959	0.9535352	Ets2	E26 avian leukemia oncogene 2, 3' domain
BG073437	0.2924538	1.016684	0.9327567	Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit
BG070254	0.2795154	1.0308769	0.8701133	Uble1b	ubiquitin-like 1 (sentrin) activating enzyme E1B
BG068357	0.2756053	1.9138117	0.7995524	AI315324	expressed sequence AI315324
BG078573	0.2726166	1.5860374	0.9018161	Usp14	ubiquitin specific protease 14
BG081033	0.2721926	1.0987934	0.6392798	Cnot7	CCR4-NOT transcription complex, subunit 7
BG079289	0.2606608	0.781505	1.1564271	2700099C18Rik	RIKEN cDNA 2700099C18 gene
BG079358	0.259395	0.9477847	0.6659272	DXImx39e	DNA segment, Chr X, Immunex 39, expressed
BG079299	0.2336673	0.3873275	0.8063483	1300019P08Rik	RIKEN cDNA 1300019P08 gene
BG063605	0.231663	0.9809958	0.6492619	Hsp86-1	heat shock protein, 86 kDa 1
BG085234	0.1930835	0.7350247	1.012302	Ywhaq	tyrosine 3-monooxygenase/tryptophan 5-

					monooxygenase activation protein, theta polypeptide
BG066056	0.1881591		0.6932494	C77863	expressed sequence C77863
BG066492	0.1061302	1.2243136	0.9320018		ESTs
BG079331	0.01		1.0290047	Pawr	PRKC, apoptosis, WT1, regulator
BG066769	0.01		0.4522683	C80276	expressed sequence C80276
BG084542	0.01	0.9754778	0.9681349	Znfn1a4	zinc finger protein, subfamily 1A, 4
BG086703	0.01	0.873849	1.0606517	2310045I 24Rik	RIKEN cDNA 2310045I24 gene
BG066233	0.01	0.7428655	2.869419	C78532	expressed sequence C78532
BG067205	0.01	0.6758663	3.178939		ESTs
BG071461	0.01	0.4972035	1.3993828		ESTs, Moderately similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG073210	0.01	1.0929809	1.6795392	AA40858 2	expressed sequence AA408582
BG068541	0.01		0.8757698		ESTs
BG085211	0.01	1.9056151	1.25057	Ell2	ELL-related RNA polymerase II, elongation factor
BG070494	0.01	0.4755175	1.0167146	Gig1	glucocorticoid-induced gene 1
BG073566	0.01	1.0121166	0.9124721		ESTs
D14540:ML L	0.01	1.118742	0.3187945		
BG080268	0.01	0.7166086	0.6956581	Cxcl1	chemokine (C-X-C motif) ligand 1
BG070205	0.01		0.7120518	MGC187 02	hypothetical protein MGC18702
Gli2a	0.01	0.8274569	0.7285465		
BG068276	0.01	0.5500649	0.8501743	1110013 H04Rik	RIKEN cDNA 1110013H04 gene
BG068420	0.01	1.2734241	0.6174294	D8Ertd5 69e	DNA segment, Chr 8, ERATO Doi 569, expressed
BG070367	0.01	1.0118917	0.6973	Trfr	transferrin receptor
BG082653	0.01	1.9298759	1.0226164	G3bp2-pending	Ras-GTPase-activating protein (GAP<120>) SH3-domain binding protein 2
BG064769	0.01	1.0679438	0.7506256	Cct5	chaperonin subunit 5 (epsilon)
BG077434	0.01	0.8763821	1.0559498	Tubb5	tubulin, beta 5

0 and 4 hour comparisons IFN a4 treatment (only Ifnar1 ko higher at 0 vs 4 hrs)

4 hours IFN a4	wt	IFNAR 1 ko	IFNAR 2 ko		
Systematic	*Normalized*	*Normalized*	*Normalized*	*Common*	*Description*
BG087448	0.91571945	0.4999562	0.60192436	Fau	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived)
BG086827	0.8537288	0.4996711	0.79061204	1500032 E05Rik	RIKEN cDNA 1500032E05 gene
BG083765	0.7983188	0.4965031	0.9140728	Tacc3	transforming, acidic coiled-coil containing protein 3
AW538365	1.8734896	0.4963809	2.096651		C0106F07-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0106F07 3', mRNA sequence.
C85066	0.5824364	0.49632215	0.73946697		ESTs
BG063729	1.3210778	0.49482942	1.7803267	Gapd	glyceraldehyde-3-phosphate dehydrogenase
BG082494	0.8975611	0.49461204	0.614805	Rnf13	ring finger protein 13
BG073474	0.9941304	0.49436495	0.913429	AI173355	expressed sequence AI173355

BG064611	1.4660816	0.49373218	1.4946579	1200008 A18Rik	RIKEN cDNA 1200008A18 gene
BG081532	0.92755556	0.4937223	1.1941884	Sfpq	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)
BG066324	1.4610894	0.49369028	1.4999241	Fgf10	fibroblast growth factor 10
BG064176	1.7962933	0.49356157	1.3779081	Lgals3	lectin, galactose binding, soluble 3
BG067364	1.3183095	0.4931016	0.6736375	Atf3	activating transcription factor 3
C76763	1.0523876	0.49263045	1.483799	1600023 E10Rik; C76763; AV03726 1	RIKEN cDNA 1600023E10 gene
BG071626	0.67932165	0.49139345	0.84910434		ESTs, Moderately similar to G3P_MOUSE Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [M.musculus]
AW555492	0.6257108	0.49040124	0.88287616		L0254C07-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0254C07 3', mRNA sequence.
BG072822	1.5256311	0.48930287	1.2236179	Lamr1	laminin receptor 1 (67kD, ribosomal protein SA)
BG067846	1.0668116	0.48917043	0.7152224	2810407 B07Rik	RIKEN cDNA 2810407B07 gene
BG083949	0.7386298	0.488452	1.9965574	Sc4mol	sterol-C4-methyl oxidase-like
BG077181	0.6317478	0.48624244	1.0374706	2010004 J23Rik	RIKEN cDNA 2010004J23 gene
BG063952	0.58208394	0.48562965	0.6742338	LOC2267 57	similar to hypothetical protein FLJ21016
AW552443	1.0792686	0.48407862	0.8451002		L0211G01-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0211G01 3', mRNA sequence.
BG087868	1.4261073	0.48360556	1.2785442	C1qb	complement component 1, q subcomponent, beta polypeptide
BG077010	1.3342658	0.4826241	1.25943	Arbp	acidic ribosomal phosphoprotein PO
BG078824	1.1284755	0.47876796	0.99494237	4930415 K17Rik	RIKEN cDNA 4930415K17 gene
BG075207	1.1123362	0.47836667	0.82552415	Rpl6	ribosomal protein L6
BG087410	0.7357062	0.47783214	0.81562006	Cd9	CD9 antigen
BG067417	1.6004249	0.4730827	1.3597223	Nfe2l2	nuclear, factor, erythroid derived 2, like 2
BG071712	2.1841235	0.46613196	1.4207867	Arl6ip	ADP-ribosylation-like factor 6 interacting protein
BG080285	0.54298556	0.46538296	1.2403728	Litaf	LPS-induced TN factor
BG086397	1.2814867	0.4653566	2.073823	P4hb	prolyl 4-hydroxylase, beta polypeptide
BG077214	0.5043963	0.46437582	0.8524133	AW5394 57	expressed sequence AW539457
BG072820	1.213599	0.4610333	1.0166085	Lamr1	laminin receptor 1 (67kD, ribosomal protein SA)
BG065313	0.81707394	0.4609571	0.74163616	Atp6v1e1	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E isoform 1
BG067012	1.4871721	0.46055135	0.7516439	Mcl1	myeloid cell leukemia sequence 1
BG066442	0.6901634	0.46020082	1.5069703	Kpna2	karyopherin (importin) alpha 2
BG077162	0.6188852	0.45863515	1.0288643	Rpl3	ribosomal protein L3
AW544184	0.79082125	0.45596898	0.76526856		C0178G03-3 NIA Mouse E7.5

					Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0178G03 3', mRNA sequence.
1HG	1.4776533	0.45474753	1.2044005		
BI076765	1.1940321	0.45455438	1.0306175		L0251D08-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0251D08 3', mRNA sequence.
BG085378	1.3177459	0.452859	1.6743897	Marcks	myristoylated alanine rich protein kinase C substrate
BG065252	0.9544186	0.45142785	0.9881122	Rnpc2	RNA-binding region (RNP1, RRM) containing 2
BG065520	0.7772316	0.44974068	0.50158066	Snrpb2	U2 small nuclear ribonucleoprotein B
BG084591	1.1255769	0.44897005	0.78025806	1500001 M02Rik	RIKEN cDNA 1500001M02 gene
BG079781	0.91382813	0.44371969	0.7262026	0610007 O07Rik	RIKEN cDNA 0610007O07 gene
BG087124	1.0228782	0.44317838	1.0565445	Fcgr3	Fc receptor, IgG, low affinity III
BG087383	0.9154859	0.44049343	1.3433744	Ctsd	cathepsin D
AW557987	2.2249365	0.4394765	1.8955398		EST, Weakly similar to D29149 proline-rich protein - mouse (fragment) [M.musculus]
BG067356	0.73677695	0.4390846	1.1622438	MGC474 04	hypothetical protein MGC47404
11HG	1.0860331	0.43791124	1.0400422		
BG080751	1.1248976	0.4352705	1.5352145	Gpi1	glucose phosphate isomerase 1
BG065875	0.72871923	0.43225008	1.1119992	2810423 O19Rik	RIKEN cDNA 2810423O19 gene
BG068616	0.9126954	0.43077806	1.2124542	Tiam2	T-cell lymphoma invasion and metastasis 2
BG073370	1.1566797	0.4275	1.1793916	Ttc3	tetratricopeptide repeat domain
BG067563	1.3057768	0.42715195	1.4875195	Npc1	Niemann Pick type C1
BG085821	0.7247637	0.42696118	1.1312243	Arbp	acidic ribosomal phosphoprotein PO
AW557547	0.9659034	0.42661852	1.042343		L0283C06-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0283C06 3', mRNA sequence.
BG087841	1.23423	0.4263092	0.8970163	Mbnl	muscleblind-like (Drosophila)
AW552118	0.6030545	0.42552567	1.1600145	D14Wsu 146e	DNA segment, Chr 14, Wayne State University 146, expressed
AA410046	1.3287435	0.424699	1.2053233	4921517 N04Rik	RIKEN cDNA 4921517N04 gene
BG086363	1.2149955	0.42429438	2.0067096	Man2b1	mannosidase 2, alpha B1
BG080767	0.8487152	0.42387655	1.0906754	Arpc5	actin related protein 2/3 complex, subunit 5 (165 kDa)
AW548498	1.4890199	0.42372134	1.4752038		L0037F10-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0037F10 3', mRNA sequence.
AW551432	1.0044826	0.42370814	1.3640963	1110007 C02Rik	RIKEN cDNA 1110007C02 gene
BG077159	1.2833623	0.4225795	1.2917907	Arpc1a	actin related protein 2/3 complex, subunit 1A (41 kDa)
BG087558	2.1522143	0.42180333	1.5440946	H2-D1	histocompatibility 2, D region locus 1
BG064783	1.1656737	0.4179851	1.0899856	Ddx5	DEAD (aspartate-glutamate-alanine-aspartate) box polypeptide 5
AW550689	1.5245591	0.41752577	1.6041796	Gpi1	glucose phosphate isomerase 1

BG088482	1.4655379	0.4151546	1.3365129	Rac2	RAS-related C3 botulinum substrate 2
AW544628	1.1234618	0.4127712	1.4191798	Itgb1	integrin beta 1 (fibronectin receptor beta)
BG072901	1.0349478	0.41168413	1.1874611	D6Ertd263e	DNA segment, Chr 6, ERATO Doi 263, expressed
BG080833	1.5622034	0.41029647	0.84973484	Oxct	3-oxoacid CoA transferase
AW549924	0.7064082	0.40388423	1.0098994		L0057E04-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0057E04 3', mRNA sequence.
BG086761	1.3004616	0.4036936	1.724975	Dscr1	Down syndrome critical region homolog 1 (human)
BG086994	1.0799673	0.40182479	1.5813507	Rab7	RAB7, member RAS oncogene family
AW553061	1.3648885	0.4006096	1.771649		L0221D02-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0221D02 3', mRNA sequence.
AW539315	1.6820024	0.39713836	1.5198036		ESTs
BG064587	0.86973184	0.39701086	0.9372198		ESTs
AW550291	0.6743542	0.39698297	0.8175881		L0062F11-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0062F11 3', mRNA sequence.
AW557342	0.70671076	0.39619964	0.8844285		L0280G03-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0280G03 3', mRNA sequence.
AW557711	1.4438368	0.39608464	1.3858343		L0285C09-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0285C09 3', mRNA sequence.
BG074109	0.612161	0.3946416	0.61811805	Hsp86-1	heat shock protein, 86 kDa 1
BG063770	1.1953427	0.39257565	1.3850878	Pabpc1	poly A binding protein, cytoplasmic 1
AW552387	0.71282035	0.39001328	1.0300883	Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
BG086439	1.143669	0.38863444	1.4667807	Anxa2	annexin A2
AW554361	0.83462137	0.38680273	1.2597696	DXHXS1008E	DNA segment, Chr X, human DXS1008E
BG077145	0.9995081	0.38677245	1.250751		H3011D05-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3011D05 5', mRNA sequence.
BG074047	1.0811977	0.38667125	1.2609892	1200015M12Rik	RIKEN cDNA 1200015M12 gene
BG068759	0.9927607	0.38540965	2.1173544	Tacc3	transforming, acidic coiled-coil containing protein 3
AW544139	0.74857235	0.3807797	0.6803019		C0178C03-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0178C03 3', mRNA sequence.
BG087516	0.98752016	0.37960103	1.4163871	Eef1a1	eukaryotic translation elongation factor 1 alpha 1
AW548883	0.8919459	0.37529647	0.62894845		L0043B06-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA

					clone L0043B06 3', mRNA sequence.
BG077677	2.2071218	0.37287062	1.4842628	Actb	actin, beta, cytoplasmic
BG064643	0.5984692	0.37138608	0.95235574	Sigirr-pending	single Ig IL-1 receptor related protein
BG064782	1.2572149	0.3692067	0.91029286	Ddx5	DEAD (aspartate-glutamate-alanine-aspartate) box polypeptide 5
BG074801	1.400487	0.3689215	1.0959604		H3139D11-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3139D11 3', mRNA sequence.
AW554113	1.2275223	0.3664078	1.8002578		EST
AW548385	0.812551	0.36402926	1.1192162		L0035H11-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0035H11 3', mRNA sequence.
BG078805	0.01	0.3594776	0.96260405	AL024069	expressed sequence AL024069
BG077678	2.2604098	0.3516896	1.6394916	Actb	actin, beta, cytoplasmic
AW553509	1.0860183	0.35041833	1.5961168		L0228B08-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0228B08 3', mRNA sequence.
BG088029	0.882713	0.34874335	0.6014261	Rpl7	ribosomal protein L7
BG072752	1.4106314	0.34774247	1.5321269	Actg	actin, gamma, cytoplasmic
BG077186	1.0492618	0.34691072	1.165817	Hspa8	heat shock 70kD protein 8
AW537151	1.4430578	0.34450853	1.9349736		G0112H12-3 NIA Mouse E7.5 Embryonic Portion cDNA Library Mus musculus cDNA clone G0112H12 3', mRNA sequence.
BG075206	1.1192656	0.3391161	0.67428386	Rpl6	ribosomal protein L6
BG071703	0.6085108	0.33555105	0.6818799	Snx5	sorting nexin 5
AW549514	0.95243776	0.330666	1.0808326		L0051G03-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0051G03 3', mRNA sequence.
BG073773	1.1157255	0.3290032	1.5567973	Eef1a1	eukaryotic translation elongation factor 1 alpha 1
BG087187	1.2144955	0.3276789	2.1322706	Ucp2	uncoupling protein 2, mitochondrial
BG068259	0.68725824	0.32581937	1.1426613	Prib	proline rich protein expressed in brain
BG080364	1.9135675	0.32115176	2.8239398	Lcp1	lymphocyte cytosolic protein 1
BG080229	1.08505	0.31860712	1.2204216	Aldo3	aldolase 3, C isoform
BG067391	1.320628	0.31846902	0.565748		H3053H09-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3053H09 3', mRNA sequence.
AU023376	0.7529591	0.3176498	1.0179406		AU023376 Mouse unfertilized egg cDNA Mus musculus cDNA clone J0431A10 3', mRNA sequence.
AW548764	1.0457134	0.31699854	1.2807021		L0041D03-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0041D03 3', mRNA sequence.
AU022819	0.6549398	0.3159203	1.1095598	LOC2267	similar to hypothetical protein

				57	FLJ21016
BG081566	3.4171438	0.31469902	0.5781187		H3066F05-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3066F05 5', mRNA sequence.
AW553356	0.70021176	0.30373934	0.89468503		EST, Weakly similar to S21369 collagen alpha 2(VI) chain precursor - mouse [M.musculus]
BG078409	0.6144986	0.30109435	0.81059784	Eno1	enolase 1, alpha non-neuron
BG078410	0.7752942	0.29686862	0.8352996	Eno1	enolase 1, alpha non-neuron
BG078361	0.8808384	0.293301	1.8260543	Eif4g2	eukaryotic translation initiation factor 4, gamma 2
C77246	1.1460378	0.29259533	0.6294068	D4Ertd100e	DNA segment, Chr 4, ERATO Doi 100, expressed
BG086285	0.9437665	0.2917961	0.93488294	Eif5a	eukaryotic translation initiation factor 5A
BG087420	0.9399523	0.29098776	1.543906	Tubb5	tubulin, beta 5
BG077307	0.79312634	0.2902645	0.7728288	Arpc5	actin related protein 2/3 complex, subunit 5 (165 kDa)
BG088028	0.6916309	0.28071433	0.5088912	Rpl7	ribosomal protein L7
C85340	3.1611109	0.27643114	2.410425	C85340	expressed sequence C85340
BG080372	2.6864214	0.25998518	0.5576892		H3052H04-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3052H04 5', mRNA sequence.
AW554346	1.4253584	0.252595	2.2853522		L0239C06-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0239C06 3', mRNA sequence.
BG086675	1.1422242	0.2512678	1.7412705	Eef2	eukaryotic translation elongation factor 2
BG073317	1.1554075	0.24288532	1.5033833		EST
AW548910	1.2015688	0.22429825	1.6916931		L0043E06-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0043E06 3', mRNA sequence.
BG063416	0.6016649	0.22360055	1.0172706	Lpl	lipoprotein lipase
AW558546	1.1171336	0.19966498	1.7134949	2310016C16Rik	RIKEN cDNA 2310016C16 gene
AW549995	0.59815437	0.19459243	0.75005656		L0058E08-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0058E08 3', mRNA sequence.
BG077072	1.7693864	0.18706526	1.3183298	Actb	actin, beta, cytoplasmic
BG066575	1.1230055	0.18588567	1.4381421	Jmj	jumonji
BG087027	1.4961755	0.18122663	1.8012018	Eef1a1	eukaryotic translation elongation factor 1 alpha 1
C78835	1.3970841	0.17232104	0.7432253	Actb	actin, beta, cytoplasmic
C79946	1.6995775	0.16986461	1.1301049	C79946	expressed sequence C79946
BG063722	1.6517607	0.15293647	1.1437354	Actb	actin, beta, cytoplasmic
BG068252		0.01	0.7965164	4930447D24Rik	RIKEN cDNA 4930447D24 gene
BG068605		0.01	1.2456515		H3067D02-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3067D02 3', mRNA sequence.

BG066695		0.01	0.71182245	C80060	expressed sequence C80060
BG066477		0.01	1.1596897	C79300	expressed sequence C79300
BG081054		0.01	1.9384314	Xlr3b	X-linked lymphocyte-regulated 3b
BG074062		0.01			ESTs
BG068332		0.01	0.7687475	Dnahc5	dynein, axonemal, heavy chain 5
C77945		0.01	0.01	D16Ertd88e	DNA segment, Chr 16, ERATO Doi 88, expressed
BG065715		0.01	0.01	D15Ertd30e	DNA segment, Chr 15, ERATO Doi 30, expressed
BG078612		0.01	0.41234392	Atpaf2	ATP synthase mitochondrial F1 complex assembly factor 2
BG068748		0.01			ESTs
C85544		0.01	2.7841656	C85544	expressed sequence C85544
BG067144		0.01	0.24553326		ESTs
BG067622		0.01	0.01	C86942	expressed sequence C86942
C77408		0.01	1.0240549	Krt2-8	keratin complex 2, basic, gene 8
BG066401		0.01	0.9230409		H3042F01-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3042F01 3', mRNA sequence.
C79533		0.01	0.82584244	C79533	expressed sequence C79533
BG083855		0.01	0.7727162	Abcg4	ATP-binding cassette, sub-family G (WHITE), member 4
BG071067		0.01	0.01	Cyp11a	cytochrome P450, 11a, cholesterol side chain cleavage
BG068471		0.01	0.01		ESTs
BG078613		0.01	0.71596485	Tm4sf10	transmembrane 4 superfamily member 10
BG065122		0.01		Enc1	ectodermal-neural cortex 1
BG068090		0.01	0.38742897	AU022052	expressed sequence AU022052
BG081458		0.01	0.34667543	D8Ertd572e	DNA segment, Chr 8, ERATO Doi 572, expressed
BG069801		0.01	0.01	2600011C06Rik	RIKEN cDNA 2600011C06 gene
BG067435		0.01	0.59147555	C86341	expressed sequence C86341
BG065311		0.01	0.17374913		ESTs, Weakly similar to Another transcription unit [Drosophila melanogaster] [D.melanogaster]
BG079815		0.01	0.71998155	Naalad2	N-acetylated alpha-linked acidic dipeptidase 2
C78858		0.01	1.7937248	2610020H08Rik	RIKEN cDNA 2610020H08 gene
BG066118		0.01	1.2910686	2810457M08Rik	RIKEN cDNA 2810457M08 gene
BG068224		0.01	0.18835102		EST
BG063865		0.01	0.47817793	1300002F13Rik	RIKEN cDNA 1300002F13 gene
BG081225		0.01	1.1454892		
BG068362		0.01	0.57178235	AA617276	expressed sequence AA617276
BG067013		0.01	0.8834957	D12Ertd364e	DNA segment, Chr 12, ERATO Doi 364, expressed
BG068570		0.01	3.4607613		H3067A03-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3067A03 3', mRNA sequence.
BG066138		0.01	15.475		ESTs

BG080227		0.01	1.1636336	2510008 M08Rik	RIKEN cDNA 2510008M08 gene
BG068691	0.01	0.01	2.4796612		H3068C09-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3068C09 3', mRNA sequence.
BG068870		0.01	2.5965493		ESTs
BG068746	1.0090349	0.01	1.6673956		H3068H05-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3068H05 3', mRNA sequence.
BG067064		0.01	0.7283598	C81557	expressed sequence C81557
BG063015		0.01	0.5558156	9430023 L20Rik	RIKEN cDNA 9430023L20 gene
BG067041		0.01	0.35643646	C81458	expressed sequence C81458
BG067323		0.01	1.4411114	C85938	expressed sequence C85938
C76512		0.01	1.5692259		C76512 Mouse 3.5-dpc blastocyst cDNA Mus musculus cDNA clone J0012C01 3', mRNA sequence.
BG063697	0.14311124	0.01			ESTs
BG066048		0.01	1.5213915	D6Ertd90 e	DNA segment, Chr 6, ERATO Doi 90, expressed
BG081142		0.01	0.31150323	MGC375 88	hypothetical protein MGC37588
BG065913		0.01	0.9578186	Lt1	lurcher transcript 1
BG080375	0.01	0.01	1.0916547	Sars2	seryl-aminoacyl-tRNA synthetase 2
BG068069		0.01	0.766551		H3061D07-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3061D07 3', mRNA sequence.
SUBQ2		0.01	0.01		
BG067571		0.01	0.89659774		H3055H02-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3055H02 3', mRNA sequence.
AW558740	4.7000003	0.01	0.86519396	Man1b	mannosidase 1, beta
BG080206		0.01	2.146797	Scg3	secretogranin III
AW552513		0.01	0.95560175		L0213F02-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0213F02 3', mRNA sequence.
BG070034	1.0259713	0.01	0.16782811		ESTs
BG072400		0.01	0.93597275		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG075162	0.77175415	0.01	0.78596884		ESTs, Weakly similar to zinc finger protein 354C; transcription factor 17-like 2 [Mus musculus] [M.musculus]
BG080895	0.24905999	0.01	2.375	AI043124	expressed sequence AI043124
BG070220	18.325	0.01	0.99063206		ESTs
C87450	0.30400902	0.01	0.94031864	AU04015 2	expressed sequence AU040152
BG068621	0.99133456	0.01	1.5949411		ESTs
BG066643		0.01	0.7591129	D1Ertd27 3e	DNA segment, Chr 1, ERATO Doi 273, expressed
BG065726	0.20444107	0.01	0.7372477	D9Ertd26 e	DNA segment, Chr 9, ERATO Doi 26, expressed
BG083170	17.05	0.01	1.1486346		ESTs, Weakly similar to I48668 zinc

					finger protein 51 - mouse [M.musculus]
AW554931	0.41767076	0.01	0.08492735		L0247E05-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0247E05 3', mRNA sequence.
BG065907	0.31498414	0.01	36.324997	C77368	expressed sequence C77368
BG066747	0.4522492	0.01		C80197	expressed sequence C80197
BG066082		0.01	0.27034828	D8Ertd82e	DNA segment, Chr 8, ERATO Doi 82, expressed
C88166	1.1001335	0.01	2.0736074		C88166 Mouse fertilized one-cell-embryo cDNA Mus musculus cDNA clone J0257H04 3', mRNA sequence.
BG067770		0.01	3.507755		ESTs, Weakly similar to L1 repeat, Tf subfamily, member 26 [Mus musculus] [M.musculus]
BG066123	0.24251461	0.01			ESTs
BG067020		0.01	1.182392	C81369	expressed sequence C81369
BG066420		0.01	0.30442202	C79133	expressed sequence C79133
BG068894	0.7354586	0.01	4.2175784	Preb	prolactin regulatory element binding
BG064062	0.01	0.01	0.26281703	Klf4	Kruppel-like factor 4 (gut)
BG080397	3.611936	0.01	0.8969531	Fmn2	formin 2
BG065894	1.4321729	0.01	1.5924457		H3036E10-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3036E10 3', mRNA sequence.
BG063989	0.8201889	0.01			ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG073990	0.26073352	0.01	0.6740819		ESTs
BG084095	0.01	0.01	8.675413	AU040377	expressed sequence AU040377
BG074267	1.1514874	0.01	1.976273		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG080386		0.01	0.3496307	C85907	expressed sequence C85907
BG072832	0.88459706	0.01	0.6957061		ESTs
BG068787	0.6888881	0.01	1.0545629		H3069C10-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3069C10 3', mRNA sequence.
BG063646	0.424698	0.01	14.199999	1810074H01Rik	RIKEN cDNA 1810074H01 gene
BG068125	2.0621655	0.01	12.446917		H3062A08-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3062A08 3', mRNA sequence.
BG068327	5.6249995	0.01	1.8781703	AI427122	expressed sequence AI427122
BG068785	0.26483482	0.01	1.225	5730421E18Rik	RIKEN cDNA 5730421E18 gene
BG069400	1.7848724	0.01	0.79103255	D9Ertd414e	DNA segment, Chr 9, ERATO Doi 414, expressed
AU022787	0.17834912	0.01	0.5672793	D7Ertd565e	DNA segment, Chr 7, ERATO Doi 565, expressed
BG066759		0.01	0.8507879	C80256	expressed sequence C80256
BG067339	0.73659605	0.01	7.298059	C88050	expressed sequence C88050
BG068585	0.97751504	0.01	1.2045829		H3067B06-3 NIA Mouse 15K cDNA

					Clone Set Mus musculus cDNA clone H3067B06 3', mRNA sequence.
BG073838	1.5597697	0.01	2.0070662		ESTs
BG066096	0.1995	0.01	3.3155298	C78024	expressed sequence C78024
BG066081	1.539532	0.01	2.8751905	C77966	expressed sequence C77966
BG074158	23.375002	0.01	1.2327504	2610001 E17Rik	RIKEN cDNA 2610001E17 gene
BG067008	0.422283	0.01	20.949999	D2Ertd52 e	DNA segment, Chr 2, ERATO Doi 52, expressed
BG068662	0.6340062	0.01	1.2243723		ESTs
BG065553	0.33792853	0.01	2.8252866	MGC364 71	hypothetical protein MGC36471
BG073492	0.28443664	0.01	0.84361666	D0H4S11 4	DNA segment, human D4S114
BG068109	1.5974408	0.01	0.8865482		H3061H02-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3061H02 3', mRNA sequence.
BG063666	0.33911216	0.01		Tex19	testis expressed gene 19
BG068172	0.5257382	0.01			ESTs
BG068504	0.3720564	0.01	1.0149044		ESTs
BG083621	1.6612965	0.01	1.8339163	Pvrl3	poliovirus receptor-related 3
BG067069	0.72154486	0.01	1.4883593	Gss	glutathione synthetase
BG073864	0.825953	0.01			ESTs
BG063600	1.0613374	0.01	1.1204958	AW5384 60	expressed sequence AW538460
BG068708	0.6754041	0.01	1.3174564		ESTs, Weakly similar to splicing factor, arginine/serine-rich 4 (SRp75); similar to splicing factor, arginine/serine-rich 4 (SFRS4) [Mus musculus] [M.musculus]
AU022932	0.7328393	0.01			ESTs
BG067318	0.48398507	0.01			ESTs, Weakly similar to Y218_HUMAN Putative deoxyribonuclease KIAA0218 [H.sapiens]
BG070793	0.53161603	0.01	1.0336246	MGC375 88	hypothetical protein MGC37588
BG070682	1.7756793	0.01	0.5150958		ESTs
BG066754	0.5661299	0.01		D2Ertd30 3e	DNA segment, Chr 2, ERATO Doi 303, expressed
C87259	0.5644156	0.01		C87259	expressed sequence C87259
C85110	0.7814918	0.01		2310050 N11Rik	RIKEN cDNA 2310050N11 gene
BG065698	0.01	0.01	0.34376073		ESTs
BG078890	0.20409198	0.01	0.8598161	Fhl2	four and a half LIM domains 2
BG080772	1.472791	0.01	1.0028852	2510008 M08Rik	RIKEN cDNA 2510008M08 gene
BG068466		0.01	0.7465491		ESTs
BG075802	0.67891073	0.01	1.2085794		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG080462	0.61944324	0.01		Pard3	par-3 (partitioning defective 3) homolog (C. elegans)
BG077527	1.1016313	0.01	1.3453732	0610013 D04Rik	RIKEN cDNA 0610013D04 gene
BG069230	0.01	0.01	9.690387	D3Ertd71	DNA segment, Chr 3, ERATO Doi

				1e	711, expressed
BG088545	0.76364213	0.01	0.9550112	Rad50	RAD50 homolog (S. cerevisiae)
BG078945	0.01	0.01	0.9279524	Nsf	N-ethylmaleimide sensitive fusion protein
BG081321	0.59153664	0.01	1.3563195	Pclo	piccolo (presynaptic cytomatrix protein)
BG066351	0.35397044	0.01	0.6377326	C78914	expressed sequence C78914
BG080749	1.483888	0.01	0.9623991	Nup153	nucleoporin 153
BG077503	0.55847996	0.01	1.3727891	Orc2l	origin recognition complex, subunit 2-like (S. cerevisiae)
C78577	2.1372175	0.01	8.05		ESTs, Weakly similar to klotho [Mus musculus] [M.musculus]
BG070907	0.8046757	0.01	0.8284702	AU017263	expressed sequence AU017263
BG068820		0.01	0.8595614	Brca2	breast cancer 2
BG067695	1.6592484	0.01	0.866569	Ube2h	ubiquitin-conjugating enzyme E2H
BG083097	0.77912956	0.01	1.2851433	AU015105	expressed sequence AU015105
BG088014	0.8593663	0.01	0.59037566	Ptprf	protein tyrosine phosphatase, receptor-type, F
BG073477	0.4272553	0.01	1.8910415		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG076494	1.1944566	0.01	2.089936	1110032N12Rik	RIKEN cDNA 1110032N12 gene
BG074724	0.57073236	0.01	1.3548003		ESTs
BG067853	0.5729594	0.01	1.1511523		H3058H10-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3058H10 3', mRNA sequence.
BG068676	0.93510586	0.01	0.94092685		ESTs
BG080029	0.68546104	0.01	1.2025602	AA939927	expressed sequence AA939927
BG068440	0.18397136	0.01	1.8100046		H3065E08-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3065E08 3', mRNA sequence.
BG083725	0.01	0.01	0.39388654		ESTs, Weakly similar to hypothetical protein FLJ11753 [Homo sapiens] [H.sapiens]
BG064228	3.1941595	0.01	1.220001	AA408199	expressed sequence AA408199
BG063176	0.19269533	0.01	0.9887814	Gjb3	gap junction membrane channel protein beta 3
BG067221	0.01	0.01	1.1459732	C85546	expressed sequence C85546
BG067494	1.0989546	0.01	1.1524255		H3055A08-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3055A08 3', mRNA sequence.
NM010824.1	1.4112808	0.01	0.7896964		
BG066997	0.26160634	0.01	0.882461	C81285	expressed sequence C81285
BG066412	0.90293723	0.01	1.3719008		ESTs, Weakly similar to A53798 58K membrane-associated protein - rat [R.norvegicus]
BG066268	1.286476	0.01	0.50390714	C78662	EST C78662
BG080326	0.4587027	0.01	1.4441761	Usp16	ubiquitin specific protease 16
BG077263	0.23905654	0.01	0.82192385	2410080	RIKEN cDNA 2410080P20 gene

				P20Rik	
BG066086		0.01	1.4920601	D1Ertd83e	DNA segment, Chr 1, ERATO Doi 83, expressed
BG080263	0.5650698	0.01	0.7349367	Mgea5	meningioma expressed antigen 5 (hyaluronidase)
BG068052	0.2296319	0.01	0.49959654		EST
BG065722	1.3089613	0.01	1.149101		H3034E03-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3034E03 3', mRNA sequence.
BG078504	0.42863393	0.01	1.1534171	Dap3	death associated protein 3
BG067099	0.34908196	0.01	0.91220546	C85133	expressed sequence C85133
BG081133	0.2910837	0.01	1.0003062	4930533H01Rik	RIKEN cDNA 4930533H01 gene
BG067112	0.39336413	0.01	3.282631	C85181	expressed sequence C85181
BG068032	0.30334046	0.01	0.9013763	AU021107	expressed sequence AU021107
BG066607	0.12009596	0.01	1.0321192	C79491	expressed sequence C79491
BG068104	0.28001174	0.01	0.3734606	AU014713	expressed sequence AU014713
BG065840		0.01	0.79533863	C77096	expressed sequence C77096
BG081448	0.31999645	0.01	20.050003	Ppp3cb	protein phosphatase 3, catalytic subunit, beta isoform
AU022521		0.01	0.09556182	AU022521	expressed sequence AU022521
BG068218	0.01	0.01	0.9581497		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG066326		0.01	0.44228458		ESTs
BG068825	0.5854873	0.01	0.01		ESTs
BG065908	1.2823169	0.01	1.1607307	C77370	expressed sequence C77370
BG068609	0.01	0.01	1.0503193		ESTs
BG068877	0.7235974	0.01	0.01		ESTs
BG067577	0.01	0.01	0.68757325	D15Ertd466e	DNA segment, Chr 15, ERATO Doi 466, expressed
BG074442	1.548526	0.01	0.59522754	Clasp1	CLIP associating protein 1
BG086203	0.830384	0.01	0.01	Hba-a1	hemoglobin alpha, adult chain 1
BG081451	0.6392775	0.01	1.0510291	AU022848	expressed sequence AU022848
C80273	0.20909448	0.01	1.6711991	C80273	expressed sequence C80273
BG083443	0.9250269	0.01	1.0391916	D2Ertd391e	DNA segment, Chr 2, ERATO Doi 391, expressed
BG068456	0.13060142	0.01	1.1048485		H3065G01-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3065G01 3', mRNA sequence.
BG067011	0.5718934	0.01	0.6570337	Col5a1	procollagen, type V, alpha 1
RLF	0.839068	0.01			
BG065071	0.42853957	0.01	2.8943903	2510004L20Rik	RIKEN cDNA 2510004L20 gene
BG063679	0.20099936	0.01	0.26596302	2810407B07Rik	RIKEN cDNA 2810407B07 gene
BG066060	1.1172125	0.01	1.587805		ESTs
BG080674	0.5282011	0.01	0.5726685		ESTs, Highly similar to hypothetical protein CG003 [Homo sapiens] [H.sapiens]
BG080402	0.88876426	0.01	7.0899453	5730409F23Rik	RIKEN cDNA 5730409F23 gene

BG065776		0.01	0.89264303	D8Ertd124e	DNA segment, Chr 8, ERATO Doi 124, expressed
BG074447	1.8931109	0.01	0.30119258		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG079828	0.01	0.01	0.61074346		Mus musculus, Similar to hypothetical protein FLJ20509, clone IMAGE:3489119, mRNA, partial cds
AW537791	0.01	0.01	0.67522025	Spnb2	beta-spectrin 2, non-erythrocytic
BG072273	0.84781986	0.01	0.92382115	Eps15	epidermal growth factor receptor pathway substrate 15
BG068431	0.01	0.01	0.8972304	DXErtd573e	DNA segment, Chr X, ERATO Doi 573, expressed
BG068451	0.01	0.01	0.6434634		H3065F08-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3065F08 3', mRNA sequence.
BG068183	0.67020124	0.01	0.5902524	AU022240	expressed sequence AU022240
BG068101	1.054028	0.01	0.37275478		ESTs
BG066134	0.01	0.01	0.8429173	Tyro3	TYRO3 protein tyrosine kinase 3
BG066775	0.59991634	0.01	0.63490146	Lrba	LPS-responsive beige-like anchor
BG067320	0.46344447	0.01	0.9782154	C85918	expressed sequence C85918
BG068767	0.01	0.01	0.6961231	1810037G04Rik	RIKEN cDNA 1810037G04 gene
BG070135	1.9762706	0.01	1.821335		ESTs
BG068789	0.6486571	0.01	0.6185415		ESTs, Weakly similar to A48998 nucleolar protein p120 - mouse (fragment) [M.musculus]
BG065303	0.9028166	0.01	0.60397613	2310010B21Rik	RIKEN cDNA 2310010B21 gene
BG068405	0.33656335	0.01	1.4835329	AU022804	expressed sequence AU022804
BG067911	2.1521566	0.01	1.0758172	2610020H15Rik	RIKEN cDNA 2610020H15 gene
BG068075	0.26272795	0.01	0.7306057	AU021880	expressed sequence AU021880
BG081536	1.0802199	0.01	1.1580994	Taf3	TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 140kDa
C79927	0.60517985	0.01	1.3826412	D4Ertd290e	DNA segment, Chr 4, ERATO Doi 290, expressed
BG068637	0.01	0.01	0.8095407		ESTs
C78385	0.56426	0.01	0.7933539	MGC36684	hypothetical protein MGC36684
BG068651	0.25760826	0.01	0.9874558		ESTs
BG065552	0.4528718	0.01	0.75283855		ESTs
BG067289	0.01	0.01	0.86205405		ESTs, Weakly similar to POL1_HUMAN Endogenous retrovirus HERV-K10 putative pol polyprotein [Includes: Reverse transcriptase ; Endonuclease] [H.sapiens]
BG068034	0.01	0.01	1.3017368	Srpk2	serine/arginine-rich protein specific kinase 2
BG070170	0.58045185	0.01	1.7633528		H3084E01-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3084E01 3', mRNA

					sequence.
BG078855	1.5873177	0.01	0.9354047	Sdccag28	serologically defined colon cancer antigen 28
BG073817	0.4207476	0.01	0.4032707		ESTs
BG065705	0.01	0.01	0.97631896	Siat7d	sialyltransferase 7 ((alpha-N-acetylneuraminyl 2,3-betagalactosyl-1,3)-N-acetyl galactosaminide alpha-2,6-sialyltransferase) D
BG068303	0.24886505	0.01	0.8320042	AU022531	expressed sequence AU022531
BG081044	0.451897	0.01	0.9437521		ESTs
BG067771	0.29878452	0.01	0.4745642		ESTs
BG067708	0.4905326	0.01	0.83196026	Bpnt1	bisphosphate 3'-nucleotidase 1
AU023920	0.01	0.01	0.9806515		ESTs
BG067705	0.49607825	0.01	0.34105718	C87198	expressed sequence C87198
BG066299	2.10879	0.01	2.17866	C78760	expressed sequence C78760
BG080992	0.01	0.01	1.1030663	2810452K22Rik	RIKEN cDNA 2810452K22 gene
BG067375	0.48070645	0.01	19.400002	C86123	expressed sequence C86123
BG066098	0.01	0.01	0.7060054		H3038H06-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3038H06 3', mRNA sequence.
BG067371	0.01	0.01	0.96268684	C86103	expressed sequence C86103
BG068804	1.2080756	0.01	0.8687064		ESTs, Moderately similar to a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 12 [Homo sapiens] [H.sapiens]
BG080700	0.89887077	0.01	0.45507595	Fn14-pending	type I transmembrane protein Fn14
BG074357		0.01	0.8735855	Psmc1	protease (prosome, macropain) 26S subunit, ATPase 1
BG069595	1.2029865	0.01	0.37026966	Cdv1	carnitine deficiency-associated gene expressed in ventricle 1
BG067464	0.9410507	0.01	2.5645142		ESTs, Weakly similar to T13B_MOUSE Tumor necrosis factor ligand superfamily member 13B (B cell-activating factor) (BAFF) [M.musculus]
BG082835	0.29021794	0.01	0.01	MGC29331	hypothetical protein MGC29331
BG067443	0.3948913	0.01	0.6582706	C86371	expressed sequence C86371
BG063665	0.5860579	0.01	1.1729563		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG065752	0.01	0.01	1.1116487		Mus musculus, clone IMAGE:4973507, mRNA
BG078814	0.33080485	0.01	0.5169226	Appbp1	amyloid beta precursor protein binding protein 1, 59kDa
BG066882		0.01	0.94316965		H3048B01-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3048B01 3', mRNA sequence.
BG081816	0.9520239	0.01	0.864252	2310032D16Rik	RIKEN cDNA 2310032D16 gene
BG073837	0.19134046	0.01	0.37481412		ESTs
C76674	0.01	0.01	0.4769209		C76674 Mouse 3.5-dpc blastocyst cDNA Mus musculus cDNA clone

					J0016D12 3', mRNA sequence.
C76153	0.01	0.01	0.9180074		C76153 Mouse 3.5-dpc blastocyst cDNA Mus musculus cDNA clone J0004F04 3', mRNA sequence.
AW554432	0.3175765	0.01	0.47616893		L0240G01-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0240G01 3', mRNA sequence.
C76493	0.01	0.01	0.56090033	C76493	expressed sequence C76493
BG078811	0.6291698	0.01	1.2408783		Mus musculus, Similar to CGI-49 protein, clone MGC:36578 IMAGE:5100552, mRNA, complete cds
BG078511	0.17415345	0.01	0.4963472	Pold2	polymerase (DNA directed), delta 2, regulatory subunit (50 kDa)
BG068380	0.01	0.01	0.7530874		ESTs
BG065270		0.01	1.1991041	D4Ertd174e	DNA segment, Chr 4, ERATO Doi 174, expressed
BG066166	0.5351158	0.01	0.06808482	D17Ertd165e	DNA segment, Chr 17, ERATO Doi 165, expressed
BG066757	0.9592049	0.01	1.2932339		ESTs, Moderately similar to ROR_HUMAN Heterogeneous nuclear ribonucleoprotein R (hnRNP R) [H.sapiens]
BG070918	0.62290525	0.01	2.0778713		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG067090	0.8738344	0.01	0.8587366		H3050E01-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3050E01 3', mRNA sequence.
BG081853	0.41707113	0.01	1.0001459	AA517451	expressed sequence AA517451
C78750	0.01	0.01	0.64949167	D13Ertd205e	DNA segment, Chr 13, ERATO Doi 205, expressed
BG067355	0.4073588	0.01	2.1928148	Poll	polymerase (DNA directed), lambda
BG069267	0.5310734	0.01	1.0010849		ESTs, Weakly similar to S60466 transposase - fruit fly (Drosophila melanogaster) transposon element S [D.melanogaster]
BG065548	0.01	0.01	0.724875	2700038G22Rik	RIKEN cDNA 2700038G22 gene
BG088920	0.7427343	0.01	1.3656032	Wee1	wee 1 homolog (S. pombe)
BG081131	0.6475138	0.01	1.2191672	Kpna4	karyopherin (importin) alpha 4
BG067367	0.5543929	0.01	1.1059402	C86090	expressed sequence C86090
BG066774	0.2521811	0.01	0.72988045	C80283	expressed sequence C80283
BG067327	0.01	0.01	0.6962304	Arl6	ADP-ribosylation-like 6
BG084858	0.01	0.01	0.01		ESTs
BG065727	0.01	0.01	3.832107	D4Ertd31e	DNA segment, Chr 4, ERATO Doi 31, expressed
BG068275	0.01	0.01	0.6929264	Rdx	radixin
BG072158	0.49624106	0.01	0.799939	BC025586	cDNA sequence BC025586
BG080607	4.7775116	0.01	1.3300817	5031415C07Rik	RIKEN cDNA 5031415C07 gene
BG080673	0.41470304	0.01	0.39643136	2310020H19Rik	RIKEN cDNA 2310020H19 gene
BG068527	0.26211438	0.01	1.5405889		ESTs

BG080490	0.01	0.01	0.6642797	1110008 J03Rik	RIKEN cDNA 1110008J03 gene
BG065088	0.24228188	0.01	0.8153519	Ddx21	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21 (RNA helicase II/Gu)
AW538503	2.3159366	0.01	1.0213039	Ugcgl	UDP-glucose ceramide glucosyltransferase-like
BG081603	0.32985657	0.01	1.1410216	1700094 M07Rik	RIKEN cDNA 1700094M07 gene
BG080514	1.0157838	0.01	1.4293534	1700058 O05Rik	RIKEN cDNA 1700058O05 gene
BG067553		0.01	0.17228581		ESTs
BG068007	1.2778021	0.01	1.6374243		ESTs
BG067759	0.01	0.01	1.2245277	C87398	expressed sequence C87398
BG068517	0.01	0.01	1.0133711		H3066D09-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3066D09 3', mRNA sequence.
BG079313	0.37983936	0.01	0.93461865	MGC369 57	hypothetical protein MGC36957
BG070564	0.798856	0.01	0.919737		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
BG081800	0.46709907	0.01	0.7971419	Rock1	Rho-associated coiled-coil forming kinase 1
BG076947	0.84385014	0.01	0.5934948	Fnbp3	formin binding protein 3
C78625	1.3643748	0.01	0.51020443		C78625 Mouse 3.5-dpc blastocyst cDNA Mus musculus cDNA clone J0052C08 3' similar to Mus musculus granzyme F gene, mRNA sequence.
BG081585	0.01	0.01	0.69854647	Pigf	phosphatidylinositol glycan, class F
BG068071	0.01	0.01	0.7867351		ESTs
BG066730	0.5723091	0.01	0.6137848	C80154	expressed sequence C80154
BG080818	0.82069236	0.01	0.90095764	Ubce8	ubiquitin-conjugating enzyme 8
BG065670	0.60564005	0.01	4.8231487	D14Ertd1 6e	DNA segment, Chr 14, ERATO Doi 16, expressed
BG067010	0.8397187	0.01	0.90935904	Hrb	HIV-1 Rev binding protein
BG068582	0.58888745	0.01	0.97094816		ESTs
BG067383	0.9066868	0.01	1.2800126		ESTs
BG078370	1.0557256	0.01	1.342956	2410004 H02Rik	RIKEN cDNA 2410004H02 gene
BG068599	0.333043	0.01	0.9257602	D11Ertd4 61e	DNA segment, Chr 11, ERATO Doi 461, expressed
BG068649	0.40985456	0.01	1.2606398		Mus musculus, Similar to KIAA1068 protein, clone IMAGE:4236345, mRNA, partial cds
BG068624	1.1106796	0.01	0.67825025		ESTs, Highly similar to regulator of nonsense transcripts 2; yeast Upf2p homolog [Homo sapiens] [H.sapiens]
BG065129		0.01	0.99104065	1500034 E06Rik	RIKEN cDNA 1500034E06 gene
BG071618	0.5552136	0.01	0.05235935		ESTs
BG068418	0.01	0.01	0.43252426		ESTs
BG068755	0.941379	0.01	2.072192	AW1079 53	expressed sequence AW107953
C85780	0.01	0.01	0.701604		C85780 Mouse fertilized one-cell-embryo cDNA Mus musculus cDNA clone J0214E04 3', mRNA

					sequence.
BG078947	0.82376903	0.01	1.898491	Lce-pending	long chain fatty acyl elongase
BG068374	0.57267267	0.01	0.7610243	D5Ertd560e	DNA segment, Chr 5, ERATO Doi 560, expressed
BG079790	1.2185645	0.01	0.8842085	Xpnpep1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble
BG081665	0.5577367	0.01	1.0575254	1110030J09Rik	RIKEN cDNA 1110030J09 gene
BG079956	0.01	0.01	0.6328215		ESTs, Weakly similar to T30081 hypothetical protein C31H2.1 - Caenorhabditis elegans [C.elegans]
BG070201	8.5	0.01	0.01	Pfdn5	prefoldin 5
BG070541	1.1187091	0.01	8.074999		ESTs
BG068343	0.70655227	0.01	0.97104865		ESTs, Moderately similar to C21124 Bkm-like sex-determining region hypothetical protein CS319 - fruit fly (Drosophila melanogaster) (fragment) [D.melanogaster]
BG080190	0.26344052	0.01	1.4120408	2310046H11Rik	RIKEN cDNA 2310046H11 gene
C85897	0.01	0.01			Mus musculus, clone IMAGE:4954162, mRNA
BG080598		0.01	0.5056731	D16Ertd454e	DNA segment, Chr 16, ERATO Doi 454, expressed
BG065537	0.1743205	0.01	1.2431564	Psmd2	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2
C77431	0.13100031	0.01	0.5928628	1810059A23Rik	RIKEN cDNA 1810059A23 gene
BG079333	0.01	0.01	1.0578666	Gdf3	growth differentiation factor 3
BG080059	0.01	0.01	0.8680682	MGC38009	hypothetical protein MGC38009
BG068322	0.4806057	0.01	0.91087186	2400003N08Rik	RIKEN cDNA 2400003N08 gene
BG063854	0.6735021	0.01	0.8109074		
BG066039	0.01	0.01	0.5199108	C77798	expressed sequence C77798
BG067678	0.51299316	0.01	0.4312876		ESTs
BG081785	0.7521446	0.01	1.2315603	Mdm2	transformed mouse 3T3 cell double minute 2
BG068615	0.01	0.01	0.84187114		ESTs
AU044228	1.001596	0.01	1.897068		EST
C81302	0.7716757	0.01	0.6541239	C81302	expressed sequence C81302
BG063298	0.8786205	0.01	0.66643125	AA408380	expressed sequence AA408380
BG070977	0.42028597	0.01	1.6268241		Mus musculus, clone MGC:8305 IMAGE:3593825, mRNA, complete cds
BG066814	0.5833691	0.01	1.7380079		H3047C03-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3047C03 3', mRNA sequence.
BG068575	0.1547016	0.01	1.5374188	Csad	cysteine sulfinic acid decarboxylase
AU043277		0.01	0.7091941	D13Ertd688e	DNA segment, Chr 13, ERATO Doi 688, expressed
BG088522	17.025002	0.01	1.298189	LOC216971	similar to hypothetical protein FLJ10700
BG067148	0.38881478	0.01	0.89688873	Fbxo5	f-box only protein 5
BG065886	0.42953703	0.01	2.9516437		ESTs

BG067004	0.5471349	0.01	0.6206703	Gata3	GATA binding protein 3
BG068403	0.01	0.01	1.0201565		ESTs
BG081674		0.01	0.6383395		H3067H01-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3067H01 5', mRNA sequence.
BG078448	0.26284313	0.01	0.57635564	Xnp	X-linked nuclear protein
BG082303	0.01	0.01	0.59395593	Dnaja3	DnaJ (Hsp40) homolog, subfamily A, member 3
BG072868	0.01	0.01	1.1166166		ESTs
BG068113		0.01	0.772833	AU023194	expressed sequence AU023194
AU044341	0.36626112	0.01	0.11964652	C87499	expressed sequence C87499
BG073923	8.475	0.01			EST
BG066088		0.01	0.38684356	D1Ertd84e	DNA segment, Chr 1, ERATO Doi 84, expressed
BG065337	0.888351	0.01	1.1000832	1110001K21Rik	RIKEN cDNA 1110001K21 gene
BG067461	0.8770018	0.01	0.55541354		ESTs
BG079368	0.47269073	0.01	0.7484852		H3038H11-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3038H11 5', mRNA sequence.
BG070917	0.86039805	0.01	0.22081959		ESTs
BG067600	0.1893974	0.01	1.1623527		ESTs
BG067906	0.54866976	0.01	0.6297452		ESTs
C77180	0.87815386	0.01	1.5043737	C77180	expressed sequence C77180
BG066748	0.39998046	0.01		C80198	expressed sequence C80198
BG067965	1.627991	0.01	0.8737698	AI788721	expressed sequence AI788721
BG070443	41.725002	0.01	1.1761147	1700086L19Rik	RIKEN cDNA 1700086L19 gene
BG077262	0.27107394	0.01	0.53629005	D1Wsu40e	DNA segment, Chr 1, Wayne State University 40, expressed
BG065745	2.7219625	0.01	1.3911649		ESTs, Moderately similar to S12207 hypothetical protein (B2 element) - mouse [M.musculus]
BG067455		0.01	0.9109639	AI314060	expressed sequence AI314060
AW537693	0.55760974	0.01	1.7590986		G0120E02-3 NIA Mouse E7.5 Embryonic Portion cDNA Library Mus musculus cDNA clone G0120E02 3', mRNA sequence.
BG065754	0.72767365	0.01	0.88654256	Ccng	cyclin G
BG067728	0.01	0.01			H3057E08-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3057E08 3', mRNA sequence.
BG080714	0.9471099	0.01	1.09817	AU021768	expressed sequence AU021768
BG067105		0.01	0.01	D2Ertd397e	DNA segment, Chr 2, ERATO Doi 397, expressed
BG068043	1.112123	0.01	1.0112307	4933400E14Rik	RIKEN cDNA 4933400E14 gene
BG068822	0.4861701	0.01	0.99188286		ESTs, Weakly similar to 2202260A puromycin sensitive aminopeptidase [Mus musculus] [M.musculus]
BG068773	0.01	0.01	0.94463444		ESTs
BG073823	0.4942906	0.01	0.01		ESTs
BG067319	0.01	0.01	1.9428009	C85907	expressed sequence C85907

BG066393	0.33455053	0.01	0.6719685	D3Ertd229e	DNA segment, Chr 3, ERATO Doi 229, expressed
BG079699	0.01	0.01	1.1064566	Cct4	chaperonin subunit 4 (delta)
BG080207	0.01	0.01	1.1112522	Il16	interleukin 16
BG081444	0.7957428	0.01	0.5471466	Tcea1	transcription elongation factor A (SII) 1
BG070629		0.01	2.225608		ESTs, Weakly similar to solute carrier family 21 (organic anion transporter), member 11 [Mus musculus] [M.musculus]
BG079795		0.01	0.9083443	Rce1	Ras and a-factor-converting enzyme 1 homolog (S. cerevisiae)
BG066480	0.88835955	0.01	0.9233355	Mint-pending	Msx2 interacting nuclear target protein
BG068106	0.4164093	0.01	1.2611822		ESTs, Moderately similar to 1614337A formin [Mus musculus] [M.musculus]
BG070579	1.0354548	0.01	0.9216211	MGC38253	hypothetical protein MGC38253
BG080272		0.01	2.245171		ESTs, Moderately similar to RIKEN cDNA 4930447D24 [Mus musculus] [M.musculus]
BG068353	0.61144555	0.01	0.92280036		ESTs
BG079620		0.01	0.6879071	Sncg	synuclein, gamma
BG063662	0.5562419	0.01	0.047565453		ESTs
BG065679	0.37435898	0.01	1.3670886	2310003C10Rik	RIKEN cDNA 2310003C10 gene
BG069782	23.175003	0.01	0.89376694	Cd63	Cd63 antigen
BG086308	0.504731	0.01	1.6825626	AW061316	expressed sequence AW061316
BG068094	1.0647528	0.01	0.8300772	D5Ertd505e	DNA segment, Chr 5, ERATO Doi 505 , expressed
BG065861		0.01	1.7076364	D19Ertd79e	DNA segment, Chr 19, ERATO Doi 79, expressed
BG066991		0.01	0.8247741	C81269	expressed sequence C81269
AU040335		0.01	0.01	Rfx2	vregulatory factor X, 2 (influences HLA class II expression)
BG064696	1.5909481	0.01	2.800124		ESTs, Weakly similar to F45E12.5.p [Caenorhabditis elegans] [C.elegans]
BG068317		0.01	1.1676242		H3064B07-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3064B07 3', mRNA sequence.
BG078134	0.339541	0.01	0.7404911	Ube2c	ubiquitin-conjugating enzyme E2C
BG069336	0.59921527	0.01		Cnot2	CCR4-NOT transcription complex, subunit 2
BG068475		0.01	0.97230726		ESTs
BG067266	0.01	0.01	0.9474463		ESTs, Weakly similar to CUB and Sushi multiple domains 1 [Mus musculus] [M.musculus]
BG071153		0.01		Dci	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenyme A isomerase)

0 and 4 hour comparisons IFN a4 treatment (only Ifnar2 ko higher at 0 vs 4 hrs)					
4 hours IFN a4	**wt**	**IFNAR 1 ko**	**IFNAR 2 ko**		
Systematic	*Normalized*	*Normalized*	*Normalized*	*Common*	*Description*
BG068830	0.62964207	0.21923676	0.49917394	1110002A21Rik	RIKEN cDNA 1110002A21 gene
BG076838	0.5283812	0.22109859	0.49815693	3830421M04Rik	RIKEN cDNA 3830421M04 gene
BI076459	0.8107792	1.0054227	0.49776152	LOC218490	similar to Transcription factor BTF3 (RNA polymerase B transcription factor 3)
BG079038		0.01	0.49594817	TRB-3	TRB-3
AW556391	1.0458742	0.752595	0.49524364	Rpl32	ribosomal protein L32
AU042918	0.51343197	0.7351262	0.4950963	Adprt1	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase) 1
BG067898	1.1157099	1.0891958	0.4937536		H3059D12-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3059D12 3', mRNA sequence.
BG082492	0.58942467	0.82851636	0.4904746	Rps18	ribosomal protein S18
BG087711	1.899008	1.2397771	0.48828563	Hdac3	histone deacetylase 3
BG070901	0.86953425	0.2224907	0.48747522	Eif1a	eukaryotic translation initiation factor 1A
BG087371	0.9276508	1.3236939	0.4863344	4931400A14Rik	RIKEN cDNA 4931400A14 gene
BG073134	1.705793	0.7751585	0.48585847		Mus musculus, Similar to retinoblastoma binding protein 1, clone IMAGE:4241494, mRNA
BG069388	1.1629523	0.29302657	0.48318505	MGC38287	hypothetical protein MGC38287
C85129	1.162309	0.7309463	0.48299667	C85129	expressed sequence C85129
BG072281	1.0649046	0.80383015	0.48259366	Ctbp1	C-terminal binding protein 1
AW543832	0.5602123	1.1629153	0.4798293	2700079K05Rik	RIKEN cDNA 2700079K05 gene
AW550178	0.90208304	1.3070395	0.47799894		L0061B04-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0061B04 3', mRNA sequence.
BG068100	0.12279948	0.35913885	0.4763078	Chic2	cysteine-rich hydrophobic domain 2
BG083522	0.63542706	0.60529304	0.47573447	1110038L14Rik	RIKEN cDNA 1110038L14 gene
BG063602	0.8207652	2.8846433	0.47534755		ESTs
BG069860	1.4240879	2.8765547	0.4749043	3110020O18Rik	RIKEN cDNA 3110020O18 gene
BG086258	0.73664784	0.73463655	0.47312045	2700060E02Rik	RIKEN cDNA 2700060E02 gene
BG076562	1.5430228		0.4719082	1110002H15Rik	RIKEN cDNA 1110002H15 gene
AW558174	0.6845755	1.9620558	0.46904078		EST
BG085864	1.2547435	0.7040134	0.4685993	Sepm-	selenoprotein M

			5	pending	
BG064304	0.9198515	0.5538239 5	0.4684764 4	Cct8	chaperonin subunit 8 (theta)
BG063927	1.3482119	0.5053547	0.4676703 5	Clic4	Chloride intracellular channel 4 (mitochondrial)
C85106	0.3816656 5	0.9962688 7	0.4670604 5	Rgs2	regulator of G-protein signaling 2
BG063667	0.6356963 5	1.0541487	0.4660288	Rps11	ribosomal protein S11
BG067793	0.6424339	0.5834308	0.465166	2010004 P11Rik	RIKEN cDNA 2010004P11 gene
BG080846	0.6624711	0.3735428 8	0.4640553	Jun	Jun oncogene
AU044574	0.9277171 5	1.0449477	0.4638671 6	3100001 N19Rik	RIKEN cDNA 3100001N19 gene
BG071514		1.209129	0.4632559 4	Crtr1- pending	Tcfcp2-related transcriptional repressor 1
BG067098	0.8124938	0.5807101	0.4622968 4	2310063 P06Rik	RIKEN cDNA 2310063P06 gene
BG068807	1.099562	0.5692193	0.4621895 6		ESTs
BI076595	0.888784	1.095855	0.4607073 7		L0002E05-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0002E05 3', mRNA sequence.
BG078810	1.8005327	0.3740061 5	0.4592777 2	Cycs	cytochrome c, somatic
BG079551	1.0181189	1.0820422	0.4566168 2	4930517 K11Rik	RIKEN cDNA 4930517K11 gene
1DR	0.5410383	0.5091263 7	0.4543298 8		
BG082424	0.6547207	0.7268693	0.4533628 5	Polr2h	polymerase (RNA) II (DNA directed) polypeptide H
BG076902	0.5584746	0.6411742	0.4520438 6	Pura	purine rich element binding protein A
BG079546	0.9804387 7		0.4497312	Ncoa6	nuclear receptor coactivator 6
BG084827	2.6806562		0.4470496 5	Igfbp4	insulin-like growth factor binding protein 4
BG063855	1.1176175	0.6323936	0.4441518	Actb	actin, beta, cytoplasmic
AW539684	0.8233865	0.9926792	0.4428329 2	Rbx1	ring-box 1
AU021534	0.9175023	0.508225	0.436909	Fen1	flap structure specific endonuclease 1
BG077300	0.5953738 7	0.3733106 3	0.4362708	3010024 O21Rik	RIKEN cDNA 3010024O21 gene
BG068236	0.01	0.01	0.4324369		ESTs
AW558391	0.7325622 4	0.5717094 5	0.4290146		L0294E03-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0294E03 3', mRNA sequence.
BG063954	0.368429	0.7779283 5	0.4242549 8	Tsn	translin
AA410137	0.7405531 4	2.3327284	0.4233915 2		EST01826 Mouse 7.5 dpc embryo ectoplacental cone cDNA library Mus musculus cDNA clone C0014E06 3', mRNA sequence.
BG077302	0.6161832	0.6946013 6	0.4221132 4	Atf7ip	activating transcription factor 7 interacting protein

AW550531	0.9254715 4	0.8304753	0.4180808 7		L0065G11-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0065G11 3', mRNA sequence.
BG072853	0.6059155 5	1.2635189	0.4119255 8	2210415 M14Rik	RIKEN cDNA 2210415M14 gene
AW538640	0.6070298 6	0.5204231	0.4110691 5		ESTs, Highly similar to SNXE_HUMAN Sorting nexin 14 [H.sapiens]
BG065142	0.7812909	1.0472156	0.4087808 7	2900028 O21Rik	RIKEN cDNA 2900028O21 gene
BG080382	1.1229372	1.9193764	0.4084153 5	5730427 N09Rik	RIKEN cDNA 5730427N09 gene
BG067456	1.1164489	3.200563	0.4074913 6	Lyzs	lysozyme
BG065113	0.6666363 5	0.3898054 4	0.4025121 3	Bcat1	Branched chain aminotransferase 1, cytosolic
BG067676	0.9075603	0.2185906 6	0.4020067	C87102	expressed sequence C87102
BG076978		0.7664345 5	0.3978901		ESTs
BG065134	0.3225119 4	0.6982546	0.3963939 2	D17H6S 56E-2	DNA segment, Chr 17, human D6S56E 2
BG063583	0.2958063 8	0.3489166	0.3947403	Slc20a1	solute carrier family 20, member 1
BG068482	0.6518704	0.8632719 5	0.3916624 5		Mus musculus, Similar to DC13 protein, clone MGC:41298 IMAGE:1548292, mRNA, complete cds
BG084290	1.350004	0.5692606	0.3879642	Idb2	inhibitor of DNA binding 2
BG063302	0.630765	1.3579123	0.3868533 7	AA40886 5	expressed sequence AA408865
BG076069	0.9217607 4	0.6912032 4	0.3776612	Cd24a	CD24a antigen
BG067314	0.6427269	1.1534672	0.3743603 8	Mrg1	myeloid ecotropic viral integration site-related gene 1
BG068084	0.5411694 6	0.9037198	0.3713823 3		ESTs, Highly similar to prefoldin 4 [Homo sapiens] [H.sapiens]
BG065683	0.01		0.3693419		ESTs
BG073126	1.1653918	0.5330481 5	0.3675535 6	Idb2	inhibitor of DNA binding 2
BG073133	0.6320718	1.7555797	0.359404		ESTs, Moderately similar to cytochrome c oxidase subunit VIIb; 1100001F07Rik [Mus musculus] [M.musculus]
BG068834	0.4733747	0.01	0.3567489 4	2610024 G14Rik	RIKEN cDNA 2610024G14 gene
BG087311	0.01	0.3028221	0.3490642		H3138B01-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3138B01 5', mRNA sequence.
BG073689	0.6912989	0.8393833 6	0.3458274 6		H3124G02-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3124G02 3', mRNA sequence.
BG077408	2.809546	1.4075199	0.3451969 3	Pmm1	phosphomannomutase 1
BG068285	0.01	0.01	0.3435943 4	AU02249 0	expressed sequence AU022490
BG064115	0.01	1.303143	0.3426157		ESTs, Moderately similar to T00334

			2		hypothetical protein KIAA0562 - human [H.sapiens]
AW550286	0.6814576	0.3142594 7	0.3337566 6	MGC305 95	hypothetical protein MGC30595
AA408929	0.8525330 4	0.4898154 4	0.3301716 7	D11Wsu 173e	DNA segment, Chr 11, Wayne State University 173, expressed
BG073053	9.936153	2.3043203	0.3217439	Ptprf	Protein tyrosine phosphatase, receptor-type, F
BG066803	0.01	1.0980352	0.3170812		H3047B03-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3047B03 3', mRNA sequence.
BG065511	0.3830189 7	0.3215154 4	0.3116997 5	Timm23	Translocase of inner mitochondrial membrane 23 homolog (yeast)
BG063581	0.5115869 6		0.3005751 4	Pdir-pending	protein disulfide isomerase-related
BG077746	1.2046113	1.4919513	0.2816666 7	Vps28	vacuolar protein sorting 28 (yeast)
BG064900	5.965598	1.4473871	0.2744545 6	Scd2	stearoyl-Coenzyme A desaturase 2
BG080379	26.750002	1.3289126	0.2449575 5	Nars	asparaginyl-tRNA synthetase
BG062933	0.9208215	0.6921308 6	0.2404227 1	Ndufs5	NADH dehydrogenase Fe-S protein 5
AU041552	0.9914200 3	0.9232482	0.2395448 4		AU041552 Mouse four-cell-embryo cDNA Mus musculus cDNA clone J1003H10 3', mRNA sequence.
BG071130	15.725001	1.0476233	0.2386045 6	Gtpbp2	GTP binding protein 2
BG084309	2.5818787	3.9462674	0.2357774 5	C030008 B15Rik	RIKEN cDNA C030008B15 gene
BG075833	1.2513359	1.5376624	0.2204803 8		ESTs
BG075074	1.2727467	1.2906297	0.2108416	Pldn	pallidin
BG069816	1.161741	1.652338	0.1773792 7		ESTs
AU016876		1.8356429	0.1159567 9		ESTs
AW554865	2.783567	1.3732293	0.0825961 6	2310047 G20Rik	RIKEN cDNA 2310047G20 gene
BG072020	0.9573998	0.7295652	0.0319786 2	Arntl	Aryl hydrocarbon receptor nuclear translocator-like
BG069806	11.107687	15.296414	0.01	Ubc	ubiquitin C
BG064847	26.550003	1.8210804	0.01	Emb	embigin

0 and 4 hour comparisons IFN a4 treatment (only wt higher4 vs 0 hrs)					
4 hours IFN a4	**wt**	**IFNAR 1 ko**	**IFNAR 2 ko**		
Systematic	*Normalized*	*Normalized*	*Normalized*	*Common*	*Description*
BG069602	67.075005	1.7497634	1.709303		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
C85808	53.84533	19.875	1.0819365	Serpina3 g	serine (or cysteine) proteinase inhibitor, clade A, member 3G
BG069953	50.649998	2.0572968	0.0262774 9		ESTs
BG080262	49.625	1.22065	1.3188295	Cib1	calcium and integrin binding 1

					(calmyrin)
BG067921	20.128523	1.3271257	0.8728625	Tgtp	T-cell specific GTPase
BG066322	12.303721	29.775	0.2907163		
BG083212	10.687444	1.6332021	1.1381179		Mus musculus, Similar to putative zinc finger protein NY-REN-34 antigen, clone MGC:29273 IMAGE:5067268, mRNA, complete cds
BG080292	8.556709	0.3907545	1.8528666		Mus musculus, Similar to KIAA1404 protein, clone IMAGE:5252426, mRNA, partial cds
BG071884	8.241115	0.9847953	0.7032125		Mus musculus, clone MGC:8305 IMAGE:3593825, mRNA, complete cds
BG076245	6.7979345	1.3356817	0.8718376	Igtp	interferon gamma induced GTPase
BG081071	6.6059647	1.0216886	1.0940069	1810015 H18Rik	RIKEN cDNA 1810015H18 gene
BG076235	6.5928345	1.0205457	1.52463	Cas1	catalase 1
BG077685	5.935183	4.7533646	0.01	Krt2-8	keratin complex 2, basic, gene 8
AU042511	5.8333364	1.2729791	0.8564529 4	Pnp	purine-nucleoside phosphorylase
BG072765	5.327126	1.315075	2.5011592		ESTs, Weakly similar to A45841 T-complex-associated-testes-expressed-1 protein - mouse [M.musculus]
BG067620	5.133399	1.054513	1.3119696		Mus musculus mVL30-1 retroelement mRNA sequence
BG070228	4.961746	0.9562602	1.1615521	Pbef-pending	pre-B-cell colony-enhancing factor
BG068328	4.951435		1.0726266	Brf2	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like
BG073726	4.9454813	0.8500783	1.0777394	Hdgfrp2	hepatoma-derived growth factor, related protein 2
BG067127	4.881085	0.6610725 5	1.5297557	Irf1	interferon regulatory factor 1
BG080607	4.7775116	0.01	1.3300817	5031415 C07Rik	RIKEN cDNA 5031415C07 gene
BG076675	4.764648	1.327667	1.4135554	Hic1	hypermethylated in cancer 1
BG088252	4.6831436	47.149998	1.4302326	Nt5c3	5'-nucleotidase, cytosolic III
BG069676	4.671795	1.7858557	0.4454098 3	AU02020 6	expressed sequence AU020206
AW551397	4.63407	1.321411	0.5188691	Col18a1	procollagen, type XVIII, alpha 1
AW538495	4.59748	0.8432517 6	0.8697958 6	2400003 B06Rik	RIKEN cDNA 2400003B06 gene
BG075133	4.5785284	1.1766694	1.1489476		ESTs, Highly similar to NPS2_MOUSE NipSnap2 protein (Glioblastoma amplified sequence) [M.musculus]
AU017390	4.5617986	1.9206673	4.220302	5830443 L24Rik	RIKEN cDNA 5830443L24 gene
AW558996	4.3366137	1.6646382	0.8227275 6		L0302G11-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0302G11 3', mRNA sequence.
BG085989	4.2779865	1.0866822	1.3416041	1110008 J21Rik	RIKEN cDNA 1110008J21 gene
BG081752	4.264439	9.616278	1.1713398		H3068F10-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone

					H3068F10 5', mRNA sequence.
BG073839	4.2103353	1.1202394	0.8394208 6	Kars	lysyl-tRNA synthetase
BG083228	4.107806	0.8772649	1.0720603	Peli1	pellino 1
BG076653	4.0917315	2.9610782	1.1893923	Bzrp	benzodiazepine receptor, peripheral
BG081632	4.014972	0.957701	1.4654877	Jak2	Janus kinase 2
BG077360	3.9377463	1.3287383	0.8462119 7	Gtf3a	general transcription factor III A
BG088340	3.9290426	1.5690056	1.6567874	1110025 J15Rik	RIKEN cDNA 1110025J15 gene
BG072303	3.866705	1.6317589	0.9285685 4		ESTs, Weakly similar to Deltex3 [Mus musculus] [M.musculus]
BG063514	3.8641322	1.7023191	0.9572412	Dag1	dystroglycan 1
BG065232	3.8087227	1.864084	0.8992966 4		Mus musculus, Similar to KIAA1404 protein, clone IMAGE:5252426, mRNA, partial cds
BG077719	3.7922285	2.0970614	1.1429751	AI44729 4	expressed sequence AI447294
BG073069	3.7759428	1.1176866	1.2078615		Mus musculus, clone IMAGE:4952607, mRNA
BG074157	3.767347	0.1667197 6	0.8035504	2810004 D21Rik	RIKEN cDNA 2810004D21 gene
BG068242	3.7270403	0.6542447	0.9173713 3	Trim30	tripartite motif protein 30
BG087414	3.7115161	6.0754075	0.9793407 3	1110018 F06Rik	RIKEN cDNA 1110018F06 gene
BG080269	3.6914442	0.9158753	1.2316436	Zfp36	zinc finger protein 36
BG064678	3.6859827	1.1964154	1.5042769	4933405 K21Rik	RIKEN cDNA 4933405K21 gene
BG087356	3.6774006	0.6432966	1.2101727	Arf4	ADP-ribosylation factor 4
BG080688	3.6611664	0.6559706	1.3335353	Csf1	colony stimulating factor 1 (macrophage)
BG069586	3.6232186	1.6805066	0.8664468	2310014 B11Rik	RIKEN cDNA 2310014B11 gene
BG067341	3.5199153	0.6982387	1.4047061		Mus musculus mVL30-1 retroelement mRNA sequence
AW546647	3.4859123	0.8553303	1.3239807	AW5466 47	expressed sequence AW546647
AU023571	3.4736578	1.2875918	1.3492008		AU023571 Mouse unfertilized egg cDNA Mus musculus cDNA clone J0434G11 3', mRNA sequence.
BG075092	3.42638	1.0108391	1.3332531		ESTs
BG081566	3.4171438	0.3146990 2	0.5781187		H3066F05-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3066F05 5', mRNA sequence.
BG067655	3.3874385	0.1590658	1.146537	Brd4	bromodomain-containing 4
BG064657	3.342294	1.0213126	1.158518	Il2rg	interleukin 2 receptor, gamma chain
BG077075	3.262386	0.3353228	1.9046127	2600002 E23Rik	RIKEN cDNA 2600002E23 gene
BG074701	3.2551756	3.805984	0.8705254	0610006 H08Rik	RIKEN cDNA 0610006H08 gene
BG084377	3.1988676	1.6234701	1.206858	1110034 C02Rik	RIKEN cDNA 1110034C02 gene
BG064652	3.1960392	1.7744862	0.9917095	Maea	macrophage erythroblast attacher
BG075778	3.1669397	0.6551152	1.0043304	Xdh	xanthine dehydrogenase
BG074295	3.1585662	1.151496	1.2483848	1200010 C09Rik	RIKEN cDNA 1200010C09 gene
BG085212	3.134084	0.9829832	0.8081440	Fcgr1	Fc receptor, IgG, high affinity I

		3	3		
BG076894	3.1039646	1.1656615	1.9190931	Mybbp1a	MYB binding protein (P160) 1a
BG075952	3.043682	1.0031345	1.5650141	AI64900 9	expressed sequence AI649009
BG073663	3.0184562	0.9594942 3	1.2160722		ESTs, Weakly similar to S1[illegible]207 hypothetical protein (B2 element) - mouse [M.musculus]
BG067897	3.0034857	0.5629180 7	1.213393	MGC287 39	hypothetical protein MGC28739
BG066309	3.002687	0.8162644	1.0302545		ESTs
BG063100	2.9928408	1.4157166	0.8026442	Cycs	cytochrome c, somatic
BG073491	2.9881217	1.5314504	0.9600469		ESTs
BG078398	2.983549	1.2182524	1.3064889	Mlp	MARCKS-like protein
BG065989	2.940885	1.0168861	0.5132342 6	C77631	expressed sequence C77631
BG068538	2.9298546	0.7988665	0.5622299 3		ESTs
BG067311	2.9149926	0.6823133	1.5152221	C85886	expressed sequence C85886
BG077554	2.9128697	1.437033	1.0957125	1810009 A16Rik	RIKEN cDNA 1810009A16 gene
BG066284	2.9041734	0.7201239 5	1.24647	Snx2	sorting nexin 2
BI078687	2.89619	0.9170961 4	1.2354647		ESTs
BG076889	2.8951452	1.556168	0.9531337 6		ESTs
BG076926	2.8837686	1.2627498	1.1222665	Pphn	pantophysin
BG083588	2.8656137	0.4999142 3	1.4212201		ESTs, Highly similar to VAB2_MOUSE Vacuolar ATP synthase subunit B, brain isoform (V-ATPase B2 subunit) (Vacuolar proton pump B isoform 2) (Endomembrane proton pump 58 kDa subunit) [M.musculus]
BG074655	2.8288891	1.7812544	1.1698256		Mus musculus, Similar to KIAA0136 protein, clone IMAGE:4511162, mRNA, partial cds
BG063922	2.828	1.0935086	1.1744928		ESTs
BG081416	2.8277535	0.7851509 5	1.3434554	G3bp2-pending	Ras-GTPase-activating protein (GAP<120>) SH3-domain binding protein 2
BG062983	2.820792	1.6411347	0.6455182	AA40999 5	expressed sequence AA409995
BG082061	2.802557	0.7341037 4	1.2389971	Gapd	glyceraldehyde-3-phosphate dehydrogenase
BG067966	2.7932267	0.3744035	1.3415219	Plod3	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3
BG073108	2.7910993	0.4638224 8	1.7352728	G7e-pending	G7e protein
BG064662	2.7863855	0.5574704	1.505613	Ppp4r1	protein phosphatase 4, regulatory subunit 1
BG067430	2.7850153	0.6354525	1.0746377	H3f3b	H3 histone, family 3B
BG075824	2.7496514	1.0155108	0.7891165 6		ESTs, Weakly similar to S24407 formin isoform IV - mouse [M.musculus]
BG083352	2.744919	0.5697335 6	0.760289		Mus musculus, Similar to hypothetical protein FLJ22693, clone IMAGE:5059780, mRNA, partial cds

AW536804	2.7440534	1.3133094	1.4893494		G0109A01-3 NIA Mouse E7.5 Embryonic Portion cDNA Library Mus musculus cDNA clone G0109A01 3', mRNA sequence.
BG085499	2.7362888	0.940671	1.0828989	Sat	spermidine/spermine N1-acetyl transferase
BG074022	2.7302384	1.2739488	1.4942331	MGC37588	hypothetical protein MGC37588
BG069095	2.7261448	1.2208871	1.0567988	Icsbp	interferon concensus sequence binding protein
BG070229	2.7249808	1.3678367	2.7036505	AU015247	expressed sequence AU015247
BI076838	2.7201958	0.7260666	1.3637992		L0291C02-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0291C02 3', mRNA sequence.
BG071583	2.7069125	1.4783293	1.5329324	Req	requiem
BG080888	2.6973996	0.72873336	1.0832807	2310047O13Rik	RIKEN cDNA 2310047O13 gene
BG080372	2.6864214	0.25998518	0.5576892		H3052H04-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3052H04 5', mRNA sequence.
BG071930	2.6722424	1.369167		1700056O17Rik	RIKEN cDNA 1700056O17 gene
AW545830	2.664577	1.1883804	1.2180785		C0198H01-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0198H01 3', mRNA sequence.
BG078470	2.65563	1.9813253	1.3387334	Rbms1	RNA binding motif, single stranded interacting protein 1
BG066650	2.647972	1.5018165	1.8642583	Psme1	proteasome (prosome, macropain) 28 subunit, alpha
BG080590	2.6467001	0.5289738	1.1723504		Mus musculus, clone IMAGE:5101040, mRNA, partial cds
BG073490	2.6275752	1.2403721	0.94930553	AU042636	expressed sequence AU042636
BG074814	2.6120644	0.51189077	1.6716322	C1qa	complement component 1, q subcomponent, alpha polypeptide
BG074910	2.6060205	0.6917969	1.6437132	Tpst1	protein-tyrosine sulfotransferase 1
BG076166	2.5909996	1.1659412	0.8455252	Ube2e1	ubiquitin-conjugating enzyme E2E 1, UBC4/5 homolog (yeast)
BG080856	2.5894744	1.0412683	0.74928993	2310037I24Rik	RIKEN cDNA 2310037I24 gene
BG072793	2.58815	0.8088612	0.01	Ly6a	lymphocyte antigen 6 complex, locus A
BG063159	2.5811882	1.2683488	0.48009664	Pvrl3	poliovirus receptor-related 3
C85660	2.5743592	1.9460727	1.5496593		ESTs
BG083664	2.5712464	1.3196783	1.3142074	Ccnl	cyclin L
BG077230	2.5641854	1.3679811	1.5420521	Pik3cd	phosphatidylinositol 3-kinase catalytic delta polypeptide
BG072743	2.5641525	0.6477635	1.1640917	Btg1	B-cell translocation gene 1, anti-proliferative
BG068652	2.5608034	0.4641044	0.6281899	Kpna3	karyopherin (importin) alpha 3
BG081598	2.5398862	0.945384	1.5473509	MGC38585	hypothetical protein MGC38585
BG081542	2.5378497	1.4123341	1.1604402	Oas1c	2'-5' oligoadenylate synthetase 1C
BG070439	2.5210865	1.0167284	0.8574021	Ncoa6ip	nuclear receptor coactivator 6

					interacting protein
BG070416	2.5116637	0.9092427	1.0193176		H3087B03-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3087B03 3', mRNA sequence.
BG073329	2.5065672	1.5872583	0.8572008 6	1110031 N17Rik	RIKEN cDNA 1110031N17 gene
BG066523	2.492862	1.3108525	1.6487195		H3043H12-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3043H12 3', mRNA sequence.
BG079475	2.4898818	1.2891058	0.7486146 7	C78549	expressed sequence C78549
BG077828	2.4786913	0.6271828	0.8983505	2610024 N24Rik	RIKEN cDNA 2610024N24 gene
BG086010	2.471094	0.8972576	1.0865096	Dncic2	dynein, cytoplasmic, intermediate chain 2
BG066899	2.457011	0.6153736	1.3385246	D4Ertd2 96e	DNA segment, Chr 4, ERATO Doi 296, expressed
BG074802	2.4328625	0.8183253	1.34528	Dncic2	dynein, cytoplasmic, intermediate chain 2
BG077824	2.4281373	0.9758058	1.1957868	D14Ertd 484e	DNA segment, Chr 14, ERATO Doi 484, expressed
BG070137	2.4250271	1.1214923	0.8575758 3	Arhgap5	Rho GTPase activating protein 5
BG071025	2.4128532	2.325257	1.5064936		EST
BG066648	2.4123795	0.7455283 4	1.0951809	D13Ertd 275e	DNA segment, Chr 13, ERATO Doi 275, expressed
BG063573	2.4071019	0.9205332	0.9328294 4	C85108	expressed sequence C85108
BG075436	2.4000113	1.2531217	1.0938232	5730461 F13Rik	RIKEN cDNA 5730461F13 gene
BG073203	2.3941064	1.2105242	1.3093032	5033428 A16Rik	RIKEN cDNA 5033428A16 gene
AW539542	2.388746	1.4819602	1.2315341	LOC234 852	similar to RIKEN cDNA 2810405I11
BG076160	2.3834002	0.9146912 7	0.9248253	Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D
BG077823	2.3827112	0.6335493	0.6308987	Cul2	cullin 2
BG063923	2.3764882	0.6564134	0.969461	H3f3b	H3 histone, family 3B
BG067078	2.375996	0.5021265	0.6951948 4	Slc21a11	solute carrier family 21 (organic anion transporter), member 11
BG087310	2.363523	0.6948546	1.4524649	1810019 E15Rik	RIKEN cDNA 1810019E15 gene
BG086663	2.3600998	0.9511904	1.8352369	Cul1	cullin 1
BG076960	2.3427508	0.4470864 2	1.0586014		H3009B09-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3009B09 5', mRNA sequence.
BG088472	2.3406558	0.9101551	1.1862571	Mapkapk 2	MAP kinase-activated protein kinase 2
BG072843	2.3373394	0.3188661 6	0.9517264 4	1200003 F12Rik	RIKEN cDNA 1200003F12 gene
BG063817	2.3315916	0.5593698	1.1011294	2010317 E03Rik	RIKEN cDNA 2010317E03 gene
BG087418	2.3311284	0.8513227 7	1.0661412	Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D
BG086411	2.3301756	2.3436525	0.5178961	Rps6kb2	ribosomal protein S6 kinase, 70kD, polypeptide 2
BG063760	2.3287647	0.8399873	1.1540028	D5Ertd1 63e	DNA segment, Chr 5, ERATO Doi 163, expressed

AU040439	2.3261495	0.7069108 5	1.309746		ESTs
BG071718	2.3190198	0.9837593 4	1.2249103		Mus musculus, clone MGC:19234 IMAGE:2648618, mRNA, complete cds
AW538503	2.3159366	0.01	1.0213039	Ugcgl	UDP-glucose ceramide glucosyltransferase-like
BG078395	2.31353	0.5911338	1.5342883	Ly6e	lymphocyte antigen 6 complex, locus E
BG082525	2.305703	3.4029424	0.5577686 4		ESTs
BG068020	2.2968097	1.1527662	0.6832681		Mus musculus, clone MGC:28609 IMAGE:4218551, mRNA, complete cds
AW550853	2.295631	1.865546	1.4220703		L0070C06-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0070C06 3', mRNA sequence.
AU044566	2.2937427	1.0574366	1.2092048	Atp6v1d	ATPase, H+ transporting, lysosomal 34kD, V1 subunit D
AU017683	2.289783	0.7572516	1.8948956		AU017683 Mouse two-cell stage embryo cDNA Mus musculus cDNA clone J0742E12 3', mRNA sequence.
BG066822	2.2882042	0.7979162	1.4015887	5730508 B09Rik	RIKEN cDNA 5730508B09 gene
BG075645	2.283179	0.5407696 4	1.3676158	Nono	non-POU-domain-containing, octamer binding protein
AW543803	2.2814052	1.7860715	0.9282837 5		C0174C08-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0174C08 3', mRNA sequence.
BG077713	2.2809975	1.5583462	0.8777398 5	Usp15	ubiquitin specific protease 15
BG077283	2.2742894	0.8056303	0.8814465	LOC233 575	similar to FGF receptor activating protein 1
BG072963	2.2717464	1.5662067	1.2926719	Dnchc1	dynein, cytoplasmic, heavy chain 1
BG077678	2.2604098	0.3516896	1.6394916	Actb	actin, beta, cytoplasmic
BG076113	2.2591217	1.1000253	0.8409405	3110040 D16Rik	RIKEN cDNA 3110040D16 gene
BG064510	2.2581306	0.5908693 7	1.8189422	Trpv2	transient receptor potential cation channel, subfamily V, member 2
BG067748	2.2580202	0.4857912 4	1.2344075		Mus musculus, clone IMAGE:4019849, mRNA, partial cds
AW538179	2.246524	2.0874734	1.2430471		C0104C07-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0104C07 3', mRNA sequence.
BG072683	2.2355087	0.7259445	1.1343275	Prnp	prion protein
AW557987	2.2249365	0.4394765	1.8955398		EST, Weakly similar to D29149 proline-rich protein - mouse (fragment) [M.musculus]
BG063518	2.2239919	0.5696671 6	1.3620986	Tcirg1	T-cell, immune regulator 1
BG087362	2.219722	0.7513477	1.4677783	Sdc4	syndecan 4
BG073141	2.2183254	1.4456055	1.2587625	Sirt1	sirtuin 1 ((silent mating type information regulation 2, homolog) 1 (S. cerevisiae)
BG077677	2.2071218	0.3728706	1.4842628	Actb	actin, beta, cytoplasmic

		2			
C77656	2.1866755	1.7138559	1.0197119	Ctsc	cathepsin C
BG071712	2.1841235	0.4661319 6	1.4207867	Ari6ip	ADP-ribosylation-like factor 6 interacting protein
BG062931	2.1795692	2.4521532	1.1226232	C1qbp	complement component 1, q subcomponent binding protein
BG066916	2.1705956	1.4434539	1.4310608		Mus musculus, clone IMAGE:4952607, mRNA
BG087666	2.161324	1.5443875	1.0154008	Psmd10	proteasome (prosome, macropain) 26S subunit, non-ATPase, 10
BG079323	2.1611261	0.9045922	1.755436	Mvp	major vault protein
BG080390	2.1586382	0.7157108	1.2506142	Rgs2	regulator of G-protein signaling 2
BG087558	2.1522143	0.4218033 3	1.5440946	H2-D1	histocompatibility 2, D region locus 1
BG067101	2.151216	0.3452744 5	1.7820269	Nucb	nucleobindin
BG067016	2.1285694	0.8217928	1.5849903	C80993	expressed sequence C80993
BG079848	2.126731	0.7303325 5	1.4585946	Jmj	jumonji
BG087454	2.118784	0.8713287	1.6244065	0610031 J06Rik	RIKEN cDNA 0610031J06 gene
BG086805	2.1149096	0.8511637 4	1.3579587	Bub1	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)
BG070458	2.1108522	0.6811600 3	0.2344365 1	1300018 P11Rik	RIKEN cDNA 1300018P11 gene
BG073636	2.1015317	1.5077125	1.2095747	Psme1	proteasome (prosome, macropain) 28 subunit, alpha
BG062932	2.097681	1.3682148	4.125		ESTs, Weakly similar to T17261 hypothetical protein DKFZp727l051.1 - human (fragment) [H.sapiens]
BG072571	2.0895236	2.1362085	1.0853227	Pi4k2a-pending	phosphatidylinositol 4-kinase type 2 alpha
BG066078	2.088002	1.0886823	1.0516322	Stx3	syntaxin 3
AW554061	2.0847359	0.7462255 4	1.5524967		L0235C08-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0235C08 3', mRNA sequence.
BG071638	2.075933	0.8820735	0.9481827	1010001 C05Rik	RIKEN cDNA 1010001C05 gene
BG076621	2.0692878	1.294003	1.546573	Hspa5	heat shock 70kD protein 5 (glucose-regulated protein, 78kD)
BG067920	2.0688045	0.7556574	1.2920161	1110003 P22Rik	RIKEN cDNA 1110003P22 gene
BG070382	2.0644145	0.667411	1.4792845		ESTs, Moderately similar to S12207 hypothetical protein (B2 element) - mouse [M.musculus]
BG073523	2.0638378	1.2437047	1.0242863	3110001I 17Rik	RIKEN cDNA 3110001I17 gene
BG086386	2.059089	0.8767271 6	1.315667	Akr1b3	aldo-keto reductase family 1, member B3 (aldose reductase)
BG066125	2.0586045	0.5335639	1.1466136	Psma4	proteasome (prosome, macropain) subunit, alpha type 4
BG063833	2.058423	1.2618893	1.4918946		H3011C05-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3011C05 3', mRNA sequence.
BG072816	2.046903	0.6677405	0.7084201	Ifi203	interferon activated gene 203
BG087169	2.0365837	1.0048559	1.1556158	P5-	protein disulfide isomerase-related

				pending	protein
BG067931	2.0337257	0.5645128 5	0.8307421		ESTs, Weakly similar to T50697 hypothetical protein KIAA0083 [imported] - human (fragment) [H.sapiens]
BG077222	2.0333152	0.9563932	1.1854222	Csk	c-src tyrosine kinase
C87546	2.0317912	0.3037369 3	0.3740411	Stk11	serine/threonine kinase 11
BG075760	2.0284297	0.7579883	1.7787533	5830428 H23Rik	RIKEN cDNA 5830428H23 gene
BG088835	2.0281484	0.9060194 5	1.5876101	Supt6h	suppressor of Ty 6 homolog (S. cerevisiae)
BG074800	2.0274367	0.9593088 6	0.9383227	Gtl6	gene trap locus 6
BG073136	2.0258665	0.9286822 7	1.5279986	Atp1b3	ATPase, Na+/K+ transporting, beta 3 polypeptide
AW553730	2.0252378	1.3348557	0.9462444	1110061 O04Rik	RIKEN cDNA 1110061O04 gene
BG074394	2.0250468	0.8287238	1.0907292	LOC213 819	similar to C7ORF12
BG067439	2.024935	0.5892664	1.6974629		Mus musculus mVL30-1 retroelement mRNA sequence
BG066678	2.012239	0.8487965	1.5268137		Mus musculus mVL30-1 retroelement mRNA sequence
BG064540	2.0109615	0.6999292 4	1.4233211	M6pr	mannose-6-phosphate receptor, cation dependent
BG076242	2.0049837	0.6137327	1.0934273		Mus musculus, clone IMAGE:4924122, mRNA
BG087986	2.0045755	0.7096697 7	1.1845939		H3147C05-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3147C05 5', mRNA sequence.
BG068295	2.0019338	1.1932136	1.3296715		ESTs

0 and 4 hour comparisons IFN a4 treatment (only Ifnar1 ko higher at 4 vs 0 hrs)					
4 hours IFN a4	**wt**	**IFNAR 1 ko**	**IFNAR 2 ko**		
Systematic	*Normalized*	*Normalized*	*Normalized*	*Common*	*Description*
BG087320	0.5971633	8.201153	1.4045954	Serpinf1	serine (or cysteine) proteinase inhibitor, clade F), member 1
BG081695	1.0282211	6.780899	1.2959772	Ftl1	ferritin light chain 1
BG075693	0.7491305	5.274269	0.1806078 3	Ddah1	dimethylarginine dimethylaminohydrolase 1
BG077818	0.8884775 6	4.567087	1.0916123	Mt1	metallothionein 1
BG085321	1.0404278	4.50316	0.8703538	Rpl13	ribosomal protein L13
AW539387	1.5070683	4.316666	0.83941		C0119C03-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0119C03 3', mRNA sequence.
AW550850	1.3230934	4.237849	0.9948182	Rpl41	ribosomal protein L41
BG068687	1.2726038	3.8478577	1.2665973	6530404 A22Rik	RIKEN cDNA 6530404A22 gene
BG063604	0.9102922	3.815381	0.8785131	Biklk	Bcl2-interacting killer-like
BG076248	1.4357599	3.7789965	1.0954458	Rpl41	ribosomal protein L41
BG062950	1.4737864	3.772765	0.9609775 5	Prep	prolyl endopeptidase

BG088791	0.8369918 5	3.7363632	0.6206739 5	2810465 O16Rik	RIKEN cDNA 2810465O16 gene
BG067083	1.4200782	3.7242193	0.875809	Sh3bgr	SH3-binding domain glutamic acid-rich protein
BG084599	0.8256933	3.7164412	0.6232523 3		H3102H01-5 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3102H01 5', mRNA sequence.
AW538729	1.0807036	3.6990778	0.9275164 6		C0111B01-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0111B01 3', mRNA sequence.
BG063749	1.3963754	3.6658535	1.1216525	2900026 A02Rik	RIKEN cDNA 2900026A02 gene
BG085522	1.1548039	3.6568227	0.8966484 7	Rps13	ribosomal protein S13
BG073693	0.8798901	3.629305	0.6709923	2410030 A14Rik	RIKEN cDNA 2410030A14 gene
BG063925	0.8963336 3	3.5455725	1.3348633	Mt2	metallothionein 2
AW554473	1.2341601	3.5282598	0.9422734 4		L0241C12-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0241C12 3', mRNA sequence.
BG067517	0.7143193	3.4171503	0.8623606	MGC474 04	hypothetical protein MGC47404
BG064173	1.3569449	3.3664482	0.908057	1810036 J22Rik	RIKEN cDNA 1810036J22 gene
BG080015	1.166373	3.324174	0.8700876	Rpl37a	ribosomal protein L37a
BG083409	0.9101999 4	3.2963245	0.6865577 7	S100a6	S100 calcium binding protein A6 (calcyclin)
BG086901	1.5830635 4	3.2399826	0.8594496 7	Rpl37a	ribosomal protein L37a (calcyclin)
BG067456	1.1164489	3.200563	0.4074913 6	Lyzs	lysozyme
AA409665	1.3119648	3.1937335	0.8214408	MGC382 87	hypothetical protein MGC38287
BG086475	1.4888581	3.1909962	0.7646345	Rps29	ribosomal protein S29
BG066675	1.099884	3.171424	0.5676538		H3044G08-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3044G08 3', mRNA sequence.
BG063738	1.3503025	3.1464076	1.0887718		ESTs
BG076314	1.2182441	3.1115518	0.6942500 5		Mus musculus, hypothetical protein, clone MGC:36488 IMAGE:5363354, mRNA, complete cds
BG080337	0.6344781 5	3.1105287	1.4043937		Mus musculus, clone MGC:11769 IMAGE:3486626, mRNA, complete cds
BG070952	1.1360743	3.0226667	0.8274913	2310024 K08Rik	RIKEN cDNA 2310024K08 gene
BG064691	1.4707295	2.9969456	0.9841765	Ppm1b	protein phosphatase 1B, magnesium dependent, beta isoform
BG066704	0.8971216 7	2.9914074	0.5848361	AL02305 1	expressed sequence AL023051
AW538688	0.5830185 4	2.9823916	0.5038202 4		C0110F03-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0110F03 3', mRNA sequence.
BG072912	1.4482623	2.97045	0.8296449	Cox7a2	cytochrome c oxidase, subunit VIIa 2
BG085382	1.171528	2.941303	0.7092845	2410030	RIKEN cDNA 2410030A14 gene

			4	A14Rik	
BG086412	1.1046097	2.8983104	0.8264864 7	Rpl31	ribosomal protein L31
BG087574	0.7144978	2.8853312	0.6072750 7	2410030 A14Rik	RIKEN cDNA 2410030A14 gene
BG063602	0.8207652	2.8846433	0.4753475 5		ESTs
BG086394	1.3887242	2.8684952	1.0431578	Rpl41	ribosomal protein L41
AW538318	0.5825369	2.812349	0.9966214		C0106A12-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0106A12 3', mRNA sequence.
AW552574	1.2682254	2.803438	1.2070423		EST
AW538759	0.6976315	2.8002958	1.0450443		EST
BG085306	0.6586292 4	2.7800019	0.5763335	Cox6c	cytochrome c oxidase, subunit VIc
AW539777	1.1943654	2.768646	0.9896178		C0124D01-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0124D01 3', mRNA sequence.
AW550294	0.8634118 4	2.7610328	0.7199597		L0062G04-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0062G04 3', mRNA sequence.
BG075284	1.39584	2.7549462	0.8849112	Rps24	ribosomal protein S24
BG084537	0.7788458	2.733436	0.7791548	Recc1	replication factor C, 140 kDa
BG082389	0.5976540 4	2.694175	0.6049382	Atp5i	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g
BG063728	1.1808703	2.6473513	1.2243643	Mrgx-pending	MORF-related gene X
BG084332	0.8919478 7	2.6407845	0.6511847 4	Rps28	ribosomal protein S28
AW538981	0.3996368	2.620024	0.6915562		C0114C01-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0114C01 3', mRNA sequence.
BG076304	0.9556239	2.6056523	1.9677548	Copeb	core promoter element binding protein
BG087018	1.2620565	2.5889182	0.6601978 5	3110005 M08Rik	RIKEN cDNA 3110005M08 gene
AW550910	1.2498443	2.5613534	0.8410203 5	Rpl31	ribosomal protein L31
BG074938	1.3675922	2.555764	0.5715492 4	0610025 G13Rik	RIKEN cDNA 0610025G13 gene
BG086335	1.2407236	2.535313	0.9720849 4	Rplp1	ribosomal protein, large, P1
BG087453	1.2773637	2.5342474	0.9260816 6	Rps23	ribosomal protein S23
BG072826	0.7965926	2.5120792	0.6804966 3	Atp5l	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g
BG075285	1.3363317	2.5026398	0.8228285	Rps24	ribosomal protein S24
BG077497	0.5916137	2.4694576	1.0308591	Sui1-rs1	suppressor of initiator codon mutations, related sequence 1 (S. cerevisiae)
BG085657	0.6124388	2.4668207	0.8671644	Rpl10a	ribosomal protein L10A
AW538610	0.6347489 4	2.4547925	0.9027098		EST, Moderately similar to AT91_MOUSE ATP synthase lipid-binding protein, mitochondrial precursor (ATP synthase proteolipid

					P1) (ATPase protein 9) (ATPase subunit C) [M.musculus]
BG077758	0.5609164	2.4419057	0.5703204	Hint	histidine triad nucleotide binding protein
BG063591	1.1213903	2.438962	0.9993371	Rpl27a	ribosomal protein L27a
BG086745	1.0247519	2.4285307	1.0124019	Ercc5	excision repair cross-complementing rodent repair deficiency,complementation group 5
BI076685	1.0767686	2.3846633	1.0202978		L0206C07-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0206C07 3', mRNA sequence.
BG077817	1.3430054	2.3788333	0.9926678	Nxf1	nuclear RNA export factor 1 homolog (S. cerevisiae)
AA410137	0.7405531 4	2.3327284	0.4233915 2		EST01826 Mouse 7.5 dpc embryo ectoplacental cone cDNA library Mus musculus cDNA clone C0014E06 3', mRNA sequence.
BG074413	1.4007332	2.3250997	0.8147554 4	Rpl37a	ribosomal protein L37a
C80078	0.8773892 5	2.3067164	0.7705328 5		ESTs
BG087061	1.3108462	2.301963	0.7198489	3200001 M24Rik	RIKEN cDNA 3200001M24 gene
BG079639	1.0755177	2.2998214	0.9431944 5	Rpl27a	ribosomal protein L27a
BG076267	1.0425047	2.2620654	0.7370512	Naca	nascent polypeptide-associated complex alpha polypeptide
BG085451	1.0909251	2.2416263	0.8342357	Marcks	myristoylated alanine rich protein kinase C substrate
BG085131	1.3063406	2.2351058	1.0465431	Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide
BG063004	1.3921765	2.221449	1.0592169	Lgals1	lectin, galactose binding, soluble 1
AW555962	0.9575119	2.2194295	0.5690632 5		L0261F12-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0261F12 3', mRNA sequence.
BG078565	1.4386263	2.2101216	0.9369603	1810004I 06Rik	RIKEN cDNA 1810004I06 gene
BG063105	1.2687117	2.1920676	0.5180597	Tapbp	TAP binding protein
AW551813	1.1818855	2.1823714	1.3309833		L0204B09-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0204B09 3', mRNA sequence.
BG078926	1.4365757	2.1643822	1.1343744	Cst3	cystatin C
BG064069	0.01	2.1507757	0.6705478		ESTs
BG086987	0.9309679	2.1477122	0.9846161 6	Sui1-rs1	suppressor of initiator codon mutations, related sequence 1 (S. cerevisiae)
BG068014	1.3125328	2.11856	0.9512108 6		Mus musculus, clone IMAGE:3586350, mRNA, partial cds
BG064078	0.9067012	2.1148975	0.8585737	2010320 M17Rik	RIKEN cDNA 2010320M17 gene
BG081194	0.5681937 3	2.110432	0.9236652	Hnrpu	heterogeneous nuclear ribonucleoprotein U
BG065203	1.1291143	2.1068904	0.9676232	Rps15	ribosomal protein S15
BG085697	1.3748252	2.0876396	1.0874193	Vps16	vacuolar protein sorting 16 (yeast)
AW549974	0.6801505	2.0636833	1.0130622		L0058C02-3 NIA Mouse E12.5

	7				Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0058C02 3', mRNA sequence.
AW548128	1.0853559	2.0594568	1.4115527		EST, Weakly similar to D29149 proline-rich protein - mouse (fragment) [M.musculus]
BG074911	0.9685564 6	2.0481799	0.9998409	Rplp1	ribosomal protein, large, P1
AW550958	0.7003760 3	2.0477154	0.8191138		L0071F08-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0071F08 3', mRNA sequence.
BG073438	0.6765952 4	2.0428584	0.5737670 7	0610025 G13Rik	RIKEN cDNA 0610025G13 gene
BG068110	0.9190543	2.0407102	0.9243054 4		ESTs
BG063750	1.4412168	2.0385513	1.5049115	2310042 M24Rik	RIKEN cDNA 2310042M24 gene
BG072476	1.1756686	2.0245094	1.0825433	Eif3s4	eukaryotic translation initiation factor 3, subunit 4 (delta, 44 kDa)
BG066861	1.4353158	2.0185075	0.7509656	D13Ertd 324e	DNA segment, Chr 13, ERATO Doi 324, expressed
BG068342	0.6818080 5	2.011381	1.2807486		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
AW543654	0.8624758	2.003626	1.1249974		C0172E09 3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0172E09 3', mRNA sequence.
BG073601	0.9671881	2.0029428	0.7446949	Dbi	diazepam binding inhibitor

0 and 4 hour comparisons IFN a4 treatment (only Ifnar2 ko higher at 4 vs 0 hrs)					
4 hours IFN a4	**wt**	**IFNAR 1 ko**	**IFNAR 2 ko**		
Systematic	*Normalized*	*Normalized*	*Normalized*	*Common*	*Description*
BG066815	2.2079396	2.088874	34.5		ESTs
BG077356	0.5814786 6	0.5317541	27.3875	1810029 G24Rik	RIKEN cDNA 1810029G24 gene
BG071582	9.325	1.69445	21.9		EST
AW549180	5	1.0348766	12.198491		EST, Weakly similar to A45964 hemoglobin alpha chain - mouse [M.musculus]
BG083088	1.5320889	2.3716304	5.4052687	Ccnd1	cyclin D1
BG070859	0.8552132	1.2641579	5.2429605	4933421 G18Rik	RIKEN cDNA 4933421G18 gene
BG082453	1.4149961	1.657507	4.8579874	Tbx19	T-box 19
BG072775	14.025001	2.3284209	4.3374014		
BG082430	0.3644428 8	0.839556	3.947909	Zfr	zinc finger RNA binding protein
BG071320	0.01	0.4526232 5	3.6890655		ESTs, Weakly similar to TC17_MOUSE Zinc finger protein 354A (Transcription factor 17) (Renal transcription factor Kid-1) (Kidney, ischemia, and developmentally regulated protein-1) [M.musculus]
BG085568	2.3302686	1.5057175	3.4775884	Cd34	CD34 antigen

BG084505	0.3531752 5	1.2621193	3.3451655	2510009 N07Rik	RIKEN cDNA 2510009N07 gene
BG072439	1.8521503	0.8634514	3.3163166	Sgk	serum/glucocorticoid regulated kinase
BG068059	0.4336812	2.0566118	3.2981656	AA40878 3	expressed sequence AA408783
BG073824	0.3783168		3.1760058	5730471 K09Rik	RIKEN cDNA 5730471K09 gene
BG077365	2.4466605	3.3132608	3.1401005	D11Ertd 153e	DNA segment, Chr 11, ERATO Doi 153, expressed
BG063420	1.3104097	0.8888762	3.1274002	AF15554 6	cDNA sequence AF155546
BG088310	1.8252496	0.5346175 4	3.1050518	Psap	prosaposin
BG067782	1.8106933	0.6165092 6	3.0397725	1110008 L16Rik	RIKEN cDNA 1110008L16 gene
BG067902	1.456096	0.9115839	2.9681664		ESTs
BG075304	2.2310266	2.070764	2.9240737	2410002 F23Rik	RIKEN cDNA 2410002F23 gene
C78321	1.6387886	1.0866147	2.891854	Ppp1r7	protein phosphatase 1, regulatory (inhibitor) subunit 7
BG086763	1.9328976	1.1881366	2.8647346	LOC218 397	similar to Ras GTPase-activating protein 1 (GTPase-activating protein) (GAP) (Ras p21 protein activator) (p120GAP) (RasGAP)
BG064649	1.1197027	1.1159912	2.8451052	Rnps1	ribonucleic acid binding protein S1
AU040926	0.9321619	2.2679203	2.8249323		AU040926 Mouse four-cell-embryo cDNA Mus musculus cDNA clone J0820H05 3', mRNA sequence.
BG080364	1.9135675	0.3211517 6	2.8239398	Lcp1	lymphocyte cytosolic protein 1
BG070774	0.9300491	1.0768957	2.811617		ESTs
BG067159	2.2538922	1.1983552	2.7996955	AI04668 1	expressed sequence AI046681
AA409347	0.9865675 6	0.9954648	2.7782822	Rpl18	ribosomal protein L18
BG066981	1.1969304	1.0735371	2.7590299		H3049C03-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3049C03 3', mRNA sequence.
BG085502	1.3204212	0.2010054 7	2.7343307	AI31431 1	expressed sequence AI314311
BG072981	0.9869523 6	3.0008624	2.7164447	1500019 G21Rik	RIKEN cDNA 1500019G21 gene
BG086363	1.5746585	0.6574374	2.7157335	Gsk3b	glycogen synthase kinase 3 beta
BG078816	0.7016105	1.1037242	2.7083278	Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
BG071426	1.5479856	1.5145643	2.6962326	ESTJ082 7E04	DNA segment, EST J0827E04
BG066667	1.6648222		2.6805913	D9Ertd2 56e	DNA segment, Chr 9, ERATO Doi 256, expressed
AU042078	1.0058699	31.024998	2.6752956	Aplp2	amyloid beta (A4) precursor-like protein 2
C77281	2.0923078	1.5932629	2.640084	Catns	catenin src
BG076546	0.9838137 6	1.9504069	2.639999	Trim8	tripartite motif protein 8
BG066360	1.5133954	0.852012	2.5777729	C78947	expressed sequence C78947
BG068561	3.2052395	0.6980489 5	2.5359063		ESTs
BG080293	1.3928728	0.4558417	2.5342903		ESTs, Moderately similar to I38902

		8			retinoblastoma binding protein RIZ - human [H.sapiens]
BG080867	1.4612935	0.4028471	2.5153408	5730504 C04Rik	RIKEN cDNA 5730504C04 gene
BG067342	0.6828050 6	1.4868207	2.4872954		ESTs, Weakly similar to TRANSPOSABLE ELEMENT TCB1 TRANSPOSASE (TRANSPOSABLE ELEMENT BARNEY TRANSPOSASE) [Caenorhabditis elegans] [C.elegans]
BG076928	5.4382043	2.0531955	2.4766588		ESTs
BG073442	0.1767100 2	0.702262	2.4661264		ESTs
BG067854	1.0088594		2.4529078		ESTs
BG065325	1.4867743	0.1442506 2	2.399072	Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)
BG063107	1.9073746	2.2211463	2.3990514	Ap3d	adaptor-related protein complex AP-3, delta subunit
BG077519	1.2182332		2.3976576	Ncl	nucleolin
BG071043	1.2452885	1.8243556	2.394085		ESTs
BG074125	1.0230272	2.720372	2.3887744	Mad4	Max dimerisation protein 4
C78014	1.3023412	2.8703485	2.3706374	D5Ertd149e	DNA segment, Chr 5, ERATO Doi 149, expressed
BG063813	0.8397615 6	1.3395896	2.358517	1810044O22Rik	RIKEN cDNA 1810044O22 gene
BG066673	1.0871384	1.8545513	2.3238585	Phgdh	3-phosphoglycerate dehydrogenase
BG066395	1.3735915	0.6628386	2.3063893	Ptpra	protein tyrosine phosphatase, receptor type, A
BG077550	1.613811	0.5000199 7	2.3039901	Tagln2	transgelin 2
BG073047	1.4782104	1.065877	2.3025072		ESTs
BG075253	0.7369912	0.6313234	2.2964537	Dgl1-pending	cardiac Abnormality/abnormal facies (CATCH22), microdeletion syndrome
BG073386	1.7670345	1.5594056	2.2886293	AW124694	expressed sequence AW124694
AU040379	1.5853883	1.1453476	2.2878375		ESTs, Weakly similar to RIKEN cDNA 5730493B19 [Mus musculus] [M.musculus]
AW554346	1.4253584	0.252595	2.2853522		L0239C06-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0239C06 3', mRNA sequence.
BG080555	0.9070691 5	0.4714556 6	2.2827919	1010001 J06Rik	RIKEN cDNA 1010001J06 gene
BG077041	1.0692046	1.3889372	2.2695398	Rap1ga1	Rap1, GTPase-activating protein 1
BG083142	1.2652667	2.3303878	2.2684429	Tex12	testis exprtessed gene 12
BG088451	1.6799873	0.4699419	2.2633708	Timp2	tissue inhibitor of metalloproteinase 2
BG066410	0.9943626 5	0.631512	2.2593493	1700105 P06Rik	RIKEN cDNA 1700105P06 gene
BG082553	4.2032166	2.9436479	2.2583904	Polr2i	polymerase (RNA) II (DNA directed) polypeptide I
BG067589	0.5148027	0.7672518 5	2.257777	Itpr5	inositol 1,4,5-triphosphate receptor 5
BG073795	2.321361	0.9994382	2.2551444		ESTs, Weakly similar to S12207 hypothetical protein (B2 element) - mouse [M.musculus]

BG069041	2.1962557	1.4489152	2.2398827	MGC29021	hypothetical protein MGC29021
BG067580	0.5005228	0.81811035	2.2269602	C86807	expressed sequence C86807
BG067245	1.2930347	0.32181838	2.2230048	2210009G21Rik	RIKEN cDNA 2210009G21 gene
BG077424	0.677867	0.1942561	2.216213	AA409702	expressed sequence AA409702
BG080352	0.982967	0.15373853	2.2093759	Selel	selectin, endothelial cell, ligand
BG072204	0.8763391	1.6176299	2.1955888	4930451C15Rik	RIKEN cDNA 4930451C15 gene
BG066641	0.5727943	0.7025778	2.189877	Scd2	stearoyl-Coenzyme A desaturase 2
AW553816	1.9464146	2.2636611	2.183782		L0232B04-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0232B04 3', mRNA sequence.
BG070885	1.7572763	1.0614195	2.1714497	2310043N10Rik	RIKEN cDNA 2310043N10 gene
BI076659	0.95008016	0.6820367	2.1663027		L0067B02-3 NIA Mouse E12.5 Female Mesonephros and Gonads cDNA Library Mus musculus cDNA clone L0067B02 3', mRNA sequence.
BG064710	1.957291	0.7276655	2.162671	Smarcf1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily f, member 1
BG086377	1.2908069	0.547618	2.1593015	P4hb	prolyl 4-hydroxylase, beta polypeptide
AW537910	1.4883399	0.72950566	2.151205		C0100H05-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0100H05 3', mRNA sequence.
AU017705	0.91295636	1.6405315	2.138767		ESTs
BG087187	1.2144955	0.3276789	2.1322706	Ucp2	uncoupling protein 2, mitochondrial
BG067299	1.0248294	0.55367154	2.1278124	C85843	expressed sequence C85843
BG064830	0.71068025	0.7640192	2.1246	MGC28623	hypothetical protein MGC28623
BG083594	1.8559039	1.18797	2.1244032	Arsdr1	androgen-regulated short-chain dehydrogenase/reductase 1
BG066773	0.79013777	0.65999407	2.1222699	B4galt6	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6
BG068759	0.9927607	0.38540965	2.1173544	Tacc3	transforming, acidic coiled-coil containing protein 3
BG064509	1.7200178	1.401889	2.1169288	Ccnf	cyclin F
BG067143	1.275159	0.7609512	2.114691	Twg-pending	twisted gastrulation protein
BG063769	0.7601722	0.35956043	2.1102514		ESTs
BG065785	5.052143	2.0524564	2.1080964	D17Ertd141e	DNA segment, Chr 17, ERATO Doi 141, expressed
AW538365	1.8734896	0.4963809	2.096651		C0106F07-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0106F07 3', mRNA sequence.
BG062938	1.9394141	1.880029	2.0963488	AA409766	expressed sequence AA409766
BG078813	1.0943782	0.9310798	2.0947309	Fsd1	fibronectin type 3 and SPRY domain-

		6			containing protein
BG076366	43.8	1.1010619	2.0833247		ESTs
BG086397	1.2814867	0.4653566	2.073823	P4hb	prolyl 4-hydroxylase, beta polypeptide
BG069229	1.1267776	1.4364237	2.072662		ESTs
BG070743	0.7045268 4	1.6803786	2.0713959		H3090F09-3 NIA Mouse 15K cDNA Clone Set Mus musculus cDNA clone H3090F09 3', mRNA sequence.
AU022611	0.4140209 3	0.6559851	2.0688915	2810489 L22Rik	RIKEN cDNA 2810489L22 gene
BG063765	2.0091734	2.1866074	2.0628257	9130427 A09Rik	RIKEN cDNA 9130427A09 gene
AA408725	1.3507144	1.0494671	2.0610015	Tuba4	tubulin, alpha 4
BG085933	0.7261963	0.7319768	2.0584545	Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)
BG076997	1.4366658	0.8530285 4	2.053847	4933439 C20Rik	RIKEN cDNA 4933439C20 gene
BG066069		0.8208438	2.050992	D1Ertd7 5e	DNA segment, Chr 1, ERATO Doi 75, expressed
C86319	1.6062586	1.5101199	2.0421636		C86319 Mouse fertilized one-cell-embryo cDNA Mus musculus cDNA clone J0224C01 3', mRNA sequence.
BG082399	1.444676	0.9932111 5	2.0413973		Mus musculus, clone MGC:36285 IMAGE:4163356, mRNA, complete cds
BG070910	1.4396268	0.5198965 7	2.0377069		ESTs
BG086699	0.8033254	1.4338346	2.0348806	1810020 M02Rik	RIKEN cDNA 1810020M02 gene
BG070380	1.0360427	0.5904951	2.0254714	Dnmt3a	DNA methyltransferase 3A
BG082314	1.7993016	1.5441452	2.0144253	Mesp2	mesoderm posterior 2
AW539112	1.2532448	0.9708895	2.007811		C0114G05-3 NIA Mouse E7.5 Extraembryonic Portion cDNA Library Mus musculus cDNA clone C0114G05 3', mRNA sequence.
BG066363	1.2149955	0.4242943 8	2.0067096	Man2b1	mannosidase 2, alpha B1
BG067766	1.3634824	1.514896	2.0053933	Nr1h2	nuclear receptor subfamily 1, group H, member 2
BG070769	0.3934421 2	0.9603215	2.0029962	AJ23758 6	hypothetical protein, clone MTA.D02.090
BG073681	0.6788378	1.0564878	2.001145	2610301 D06Rik	RIKEN cDNA 2610301D06 gene

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