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SOCS3 regulation by microRNAs, cytokines and HCV



A thesis submitted to the University of Dublin for the degree of
Doctor of Philosophy

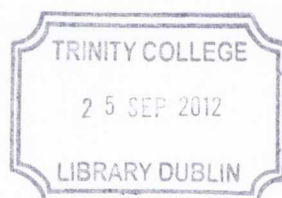
2012

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Thesis 9663

Declaration

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Poster Presentations

1. **Aideen Collins**, Nollaig Bourke, John E. Hegarty, Nigel J. Stevenson, Cliona O'Farrelly. 2009. Differential expression of suppressor of cytokine signalling (SOCS) in peripheral blood mononuclear cells (PBMCs) of hepatitis c virus (HCV) infected patients. *16th International symposium for Hepatitis C and related viruses, Nice, France.*
2. **Aideen Collins**, Nollaig Bourke, John E. Hegarty, Nigel J. Stevenson, Cliona O'Farrelly. 2009. Differential expression of suppressor of cytokine signalling (SOCS) in peripheral blood mononuclear cells (PBMCs) of hepatitis c virus (HCV) infected patients. *Irish Society of Immunology annual meeting: 'Regulation of the immune system in health and disease', Dublin, Ireland.*
3. **Aideen Collins**, Nollaig Bourke, John E. Hegarty, Nigel J. Stevenson, Cliona O'Farrelly. 2009. Differential expression of suppressor of cytokine signalling (SOCS) in peripheral blood mononuclear cells (PBMCs) of hepatitis c virus (HCV) infected patients. *Hepatitis C - 3rd International Conference, Dublin, Ireland.*
4. **Aideen Collins**, Nollaig Bourke, John E. Hegarty, Nigel J. Stevenson, Cliona O'Farrelly. 2010. Elevated Suppressor of Cytokine Signalling (SOCS) 3 in Peripheral Blood Mononuclear Cells (PBMCs) of Hepatitis C Virus (HCV) patients inhibits further induction by Interferon (IFN)- α . *EMBO Workshop: Viruses and innate immunity, Science Gallery, Dublin, Ireland.*
5. **Aideen Collins**, Nollaig Bourke, John E. Hegarty, Nigel J. Stevenson, Cliona O'Farrelly. 2010. Elevated Suppressor of Cytokine Signalling (SOCS) 3 in Peripheral Blood Mononuclear Cells (PBMCs) of Hepatitis C Virus (HCV) patients inhibits further induction by Interferon (IFN)- α . *3rd School of Medicine Postgraduate Research Day, St. James's Hospital, Dublin, Ireland.*
6. **Aideen Collins**, Nollaig Bourke, John E. Hegarty, Nigel J. Stevenson, Cliona O'Farrelly. 2010. Decreased microRNA (miRNA) expression may be responsible for enhanced suppressor of cytokine signalling (SOCS) levels in Hepatitis C Virus (HCV) infected patients. *RNAi and miRNA Europe, The Burlington Hotel, Dublin 4, Ireland.*

7. **Aideen Collins**, Nollaig Bourke, John E. Hegarty, Nigel J. Stevenson, Cliona O'Farrelly. 2010. Elevated Suppressor of Cytokine Signalling (SOCS) 3 in Peripheral Blood Mononuclear Cells (PBMCs) of Hepatitis C Virus (HCV) patients inhibits further induction by Interferon (IFN)- α . *ISI 25th Anniversary Symposium, Science Gallery, Dublin, Ireland*.

Abbreviations

µg:	microgram
µl:	microliter
2'-5'OAS:	2'-5' oligoadenylate synthetase
ALT:	Alanine aminotransferase
AMP:	Anti-microbial peptide
APCs:	Antigen presenting cells
ASC:	Apoptosis-associated speck-like protein containing a CARD
B2M:	β2 microglobulin
BCA:	Bicinchoninic acid
BIM:	Bcl-2-like protein 11
BSA:	Bovine serum albumin
CARD:	Caspase activation and recruitment domain
Cardif:	CARD adaptor inducing IFN-β
CCND:	Cyclin D1
CD81:	Cluster of differentiation 81
cFLIP:	cellular FLICE-inhibitory protein
CML:	Chronic myelogenous leukemia
CNTF:	Ciliary neurotrophic factor
COX2:	Cyclooxygenase type 2
CTL:	Cytotoxic T lymphocyte
CXCL10:	Chemokine C-X-C motif chemokine 10
CYLD:	Cylindromatosis tumour suppressor
DAI:	DNA-dependent activator of IFN-regulatory factor
DAP:	DiAminoPimelic acid
DCs:	Dendritic cells
DD:	Death domain
DMEM:	Dulbecco's modified eagles medium
Ds:	Double stranded

DUB:	de-ubiquitinating
ECS:	Elongin C–Cullin–SOCS box
EPO:	Erythropoietin
ER:	Endoplasmic reticulum
EV:	Empty vector
Exp5:	Exportin 5
FADD:	Fas-associated protein with DD
FCS:	Fetal calf serum
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GAS:	IFN- γ -activated site
G-CSF:	Granulocyte colony-stimulating factor
GH:	Growth Hormone
G-MCSF:	Granulocyte macrophage colony-stimulating factor
gp-130:	Glycoprotein 130
GUSB:	beta glucuronidase
HCC:	Hepatocellular carcinoma
HCV:	Hepatitis C Virus
HEK:	Human embryonic kidney cell line
HIN:	Hematopoietic interferon-inducible nuclear antigens
HIV:	Human immunodeficiency virus
HMNCs:	Hepatic mononuclear cells
HSV-1:	Herpes simplex virus type 1
HUH7:	Human hepatoma cell line 7
HVR-1:	Hypervariable region 1
IBD:	Inflammatory bowel disease
ICAM-1:	Intercellular adhesion molecule 1
IFI16:	Interferon inducible protein 16
IFITM:	IFN-inducible transmembrane
IFN- α :	Interferon-alpha

IFNAR:	IFN- α receptor
IFNGR:	IFN- γ receptor
Ig:	Immunoglobulin
IGF-1:	Insulin-like growth factor 1
IKK:	Inhibitory κ B kinase
IL:	Interleukin
IL-1RAcP:	IL-1 receptor accessory protein
iNOS:	inducible nitric oxide synthase
IPS-1:	IFN- β promoter stimulator
IRAK:	IL-1 receptor-associated kinase
IRES:	Internal ribosomal entry site
IRF:	Interferon regulatory factor
ISDR:	Interferon sensitivity determining region
ISGs:	IFN stimulated genes
ISGF3:	IFN-stimulated gene factor
ISRE:	IFN-sensitive response elements
JAK:	Janus kinase
JFH-1:	Japanese fulminant hepatitis 1
JNK:	c-Jun N-terminal kinases
KEGG:	Kyoto encyclopedia of genes and genomes
KIR:	Kinase inhibitory region
KO:	Knockout
LDL:	Low-density lipoprotein
LFA:	Lymphocyte function-associated antigen
LGP2:	Laboratory of genetics and physiology 2
LIF:	Leukemia inhibitory factor
LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeat
Mda5:	Melanoma differentiation-associated antigen 5

mDC:	Myeloid dendritic cell
MDP:	Muramyl DiPeptide
MHC:	Major histocompatibility complex
min:	minutes
miRNAs:	microRNAs
ml:	millilitre
MM:	Multiple myeloma
MMNC:	Mature microRNA negative control
mRNA:	messenger ribonucleic acid
MS:	Multiple sclerosis
mTOR:	mammalian target of rapamycin
MxA:	Myxovirus A
MyD88:	Myeloid Differentiation factor 88
NAIP:	NLR family apoptosis inhibitory protein
NALP:	NACHT-LRR-pyrin domain (PYD)-containing protein
NCR:	Non coding region
NF- κ B:	Nuclear factor κ B
ng:	nanogram
NK:	Natural killer
NLRs:	Nod-like receptors
nM:	nanomolar
NO:	Nitric oxide
NS:	Non-structural
ORF:	Open reading frame
PAMPs:	Pathogen-associated molecular patterns
PBMCs:	Peripheral blood mononuclear cells
PBST:	Phosphate buffered saline + tween
PEST:	Pro, glutamic acid (E), Ser and Thr motif
PGE2:	Prostaglandin-E2

PI3K:	Phosphoinositide-3-kinase
PIAS:	Protein inhibitors of activated STATs
PKR:	Protein kinase R
PPIA:	Peptidylprolyl isomerase A/Cyclophilin A
PRRs:	Pattern recognition receptors
PSGL:	P-selectin glycoprotein ligand
PTEN:	Phosphatase tensin homolog
PTK:	Protein tyrosine kinase
PVDF:	Polyvinylidene difluoride
PYD:	Pyrin domain
PYHIN:	PYD and HIN domain family member
qRT-PCR:	quantitative real time PCR
RA:	Rheumatoid arthritis
RIBA:	Recombinant immunoblot assay
RIG-I:	Retinoic-acid-inducible protein 1 like receptors
RIP-1:	Receptor interacting protein 1
RIPA:	Recombinant immunoblot assay
RISC:	RNA induced silencing complex
RLRs:	RIG-I-like receptors
RPMI:	Roswell Park Memorial Institute
RPS15:	Ribosomal protein 15
SD:	Standard deviation
SDS-PAGE:	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
SH-2:	Src homology 2
SHP-1:	SH2-containing phosphatase-1
SIAH:	Seven in absentia homolog
SLE:	Systemic lupus erythematosus
SLPI:	Secretory leukocyte protease inhibitor
SOCS:	Suppressors of cytokine signalling

SPRED2:	Sprouty-related EVH1 domain-containing protein 2
SPRY:	Sprouty homolog 2
SR-BI:	Scavenger receptor class B1
Ss:	Single stranded
STAM:	Signal transducing adapter molecule
STAT:	Signal transducer and activator of transcription
STING:	Stimulator of IFN gene
SUMO:	Small ubiquitin-like modifier
SVR:	Sustained viral response
TAB-1:	TAK1-binding protein 1
TAK-1:	Transforming growth factor (TGF)- β activated-kinase
TBK-1:	Serine/threonine-protein kinase 1
TGF- β :	Transforming growth factor- β
TIR:	Toll/interleukin 1 receptor
TLRs:	Toll like receptors
TNF- α :	Tumour Necrosis Factor- α
TNFR:	TNF receptor
TRADD:	TNF Receptor-Associated Death Domain
TRAF2:	TNFR-associated factor-2
TRIF:	TIR-domain-containing adaptor inducing IFN- β
TSH:	Thyroid stimulating hormone
TSLP:	Thymic stromal lymphopoietin
TYK-2:	Tyrosine kinase 2
UTR:	Untranslated region
VCAM-1:	Vascular cell adhesion molecule 1
WT:	Wild type

Abstract

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is the principal signalling mechanism for a wide array of cytokines. Dysregulation of JAK-STAT pathway has been noted in cancer and chronic viral disease. Suppressors of cytokine signalling (SOCS) proteins negatively regulate the JAK-STAT pathway by inhibiting JAKs and STATs and/or targeting them for proteasomal degradation. Although SOCS are important regulators of the JAK-STAT pathway, their own regulation has not been fully investigated and is the focus of this thesis.

microRNA (miR)-19a was reported to be induced by interferon (IFN)- α which signals through the JAK-STAT pathway. I hypothesised that miR-19a could regulate JAK-STAT signalling, possibly by controlling SOCS expression. Twelve JAK-STAT pathway proteins contained binding sites for miR-19a and I investigated the functional effects of four. SOCS3 expression was significantly inhibited by miR-19a, which resulted in increased IFN- α and interleukin (IL)-6-mediated phosphorylation of STAT3, demonstrating its regulatory effect upon the JAK-STAT pathway.

Hepatitis C virus (HCV) has developed successful immune evasion mechanisms and there is evidence to suggest that HCV induces SOCS3. In peripheral blood mononuclear cells (PBMCs) from healthy and HCV infected individuals, I analysed SOCS expression and induction in response to IFN- α . I found basal SOCS3 mRNA levels to be significantly elevated in HCV+ PBMCs, compared to healthy controls. SOCS3 was up-regulated in response to IFN- α . SOCS3 levels were also elevated in the hepatocyte cell line, Huh7, expressing HCV and a similar pattern of induction was observed.

Chronic HCV infection can remain undetected for many years, often inflicting no detectable or mild pathology. The tumour necrosis factor (TNF)- α /NF- κ B signalling pathway has been shown to be regulated by SOCS proteins, and I wondered whether HCV induced SOCS3 to down-regulate TNF- α pro-inflammatory signalling. To investigate this I analysed TNF- α -induction of pro-inflammatory cytokines, and I κ B degradation, in the PBMCs from healthy and HCV infected individuals. In response to TNF- α , I found I κ B degradation to be partially inhibited and IL-6, CXCL8 and TNF- α had reduced levels of induction in HCV+ PBMCs. I also expressed HCV in Huh7 cells and observed a significant reduction of CXCL8. To correlate this with HCV-induced

SOCS3, expression of SOCS3 was silenced using shRNA and I observed restoration of TNF- α -induced CXCL8, indicating that HCV-induced SOCS3 specifically inhibited TNF- α induction of CXCL8.

In this project I have identified a novel regulator of SOCS3, miR-19a, which will improve our knowledge of the control and function of this protein. I have also uncovered how HCV exploits SOCS3 to down-regulate the TNF- α /NF- κ B signalling cascade, thereby limiting pathology in the host and promoting its own survival. These findings may contribute to the understanding of inflammatory diseases associated with aberrant SOCS3 expression, and to the possibility of a therapeutic strategy aimed at reducing SOCS3 levels during HCV infection.

Chapter 1

General Introduction

CHAPTER 1: INTRODUCTION

Our immune system is a highly effective defence force employed to seek out and destroy anything foreign or dangerous that enters, or is generated by, our system. It is designed to protect from eukaryotic parasites, viruses, bacteria, fungi and other pathogens that endeavour to invade our body. It is comprised of many interdependent cell types, and their molecular products, specialised in appearance and function but collectively have a similar goal, to protect the body. An important function of the immune system is the ability to distinguish between self and non-self. The immune cells can engulf bacteria, detect viruses and virally infected cells, and kill tumour cells with the help of secreted proteins. Cytokines are small molecules secreted by specific cells of the immune system which carry signals locally between cells, and thus stimulate cell responses. They include ILs, growth hormones and cell signal molecules, which trigger inflammation and respond to infections. The immune system can be divided into two major subdivisions known as innate and adaptive immunity. Innate immunity is also referred to as the first line of defence, as it comes into play within hours of detecting an antigen. Adaptive immunity refers to an antigen-specific immune response targeted specifically at the invading pathogen, with an ability to protect against re-exposure. The latter can take days to develop, so we depend on the innate immune system to survive until the more complex and long-term process kicks in.

1.1 Innate Immunity

Innate immunity is the primary defence system against invading pathogens. A major component of the innate immune system is barrier defence, such as the skin, gastric, genital and respiratory mucosa and stomach acid, which prevent the pathogen from getting into the body. If an infective agent succeeds in gaining entry into the body, it

faces detection by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), on the surface of immune cells, followed by ingestion or destruction by anti-microbial peptides (AMPs). Macrophages are phagocytic white blood cells that kill engulfed microbes by producing toxic chemicals and powerful degradative enzymes, for example, lysozyme. An important side effect of this process is the release of cytokines and chemokines into the surrounding tissue which initiates inflammation. Inflammation is a key component of the normal immune response to viruses, bacteria, parasites and cancer.

1.2 Inflammation

Inflammation is a localised protective reaction of tissue to irritation, injury or infection, which results in the hallmark clinical symptoms of redness, pain, heat and swelling, first described by Celsus in the 1st century CE. Redness and heat are due to vessel dilation and increased blood flow, pain is due to pressure on nerve endings and the direct effect of chemical mediators released during the inflammatory response, and swelling is due to fluid leaking out of the vessels (oedema). The purpose of the inflammatory response is to contain and eradicate the invading pathogen, initiate the immune response and to promote tissue healing [1]. Inflammation is characterised by several basic mechanisms including:

1. **Vasodilation** of the local blood vessels occurs when smooth muscles within arterioles relax. Initial vasoconstriction is followed by a change in calibre and flow of blood to tissue site. Capillaries and venules also dilate.
2. **Increased permeability** of the capillaries is due to endothelial cells contracting while the inter-cell junctions retract. This allows leakage of fluid, made up of proteins and cellular contents, into the interstitial space.

3. **Accumulation of leukocytes** at the site of infection begins when white blood cells migrate out of the blood vessel and tumble along epithelial surfaces. Adhesion molecules on the surface of endothelial cells attach to integrins on the surface of leukocytes, allowing them to squeeze through the space between endothelial cells.
4. **Chemotaxis and phagocytosis** results in migration of granulocytes and monocytes along chemotactic gradients into tissue. Cells recognise and engulf the organism resulting in the release of lysozyme, capable of breaking down cells and extracellular matrix material, and subsequent death of the pathogen. Leukocytes can generate active peroxide and hydrogen peroxide radicals which upon phagocytosis cause an oxidative burst, resulting in the oxidation of microbial proteins and disruption of the cell wall.
5. **Coagulation** is induced by tissue damage and is caused by fibrin clots which form to localise the infection and stop bleeding.
6. **Repair and healing** is initially mediated by macrophages which clear out any bacteria and foreign bodies in the infected area. Neutrophils release cytokines which leads to the creation of new blood vessels while fibroblasts deposit collagen, which acts as a framework for new cells. This is referred to as granulation tissue [2].

Inflammation is triggered and enhanced by a number of chemical inflammatory mediators summarised in Table 1.1.

Types of mediators	Example	Origin	Functions	Ref.
Vasoactive amines	Histamine, serotonin	Mast cells, basophils, platelets	Promotes contraction of smooth muscles, dilation of arterioles, contraction of endothelial cells, increased vascular permeability	[3]
Plasma kinins	Bradykinin	Plasma derived	Increases vascular permeability, contraction of smooth muscle, dilation of vessels, activation of clotting factors, chemical mediator of pain	[4]
Complement system	C5a, C3a, C3b, C5b	Enzyme activated	Cause white blood cells to release mediators, vascular permeability, chemotaxis, cell lysis	[5]
Arachidonic acid metabolites	Prostaglandins, leukotrienes	Cell membrane phospholipids	Vascular permeability. Vascular constriction, leukocyte chemotaxis, potentiates other mediators	[2]
Cytokines and chemokines	IL-1, TNF- α , CXCL8	White blood cells	Induce second phase of inflammatory process, control duration and amplitude of signals, activate macrophages, stimulate collagen and collagenase prod. by fibroblasts, chemotaxis, trigger blood-clotting cascade	[6]
Clotting system	Fibrinopeptides, fibrinolytics	Plasma proteins	Permeability, chemotactic for leukocytes, generation of fibrin, generation of plasmin	[7]
Nitric oxide	NO	Endothelial cells, macrophages	Vasodilator, anti-platelet aggregation, helps with macrophage anti-microbial activity	[8]

Table 1.1: Mediators and their role in inflammation

Epithelial cells are exposed to many pathogens and their activation can trigger immediate host defence mechanisms. Epithelial cells release vast quantities of pro-inflammatory cytokines, growth factors and chemokines, resulting in the amplification of eosinophils, basophils and other inflammatory cells [9]. During the initial stages of inflammation, leukotrienes, prostaglandins and histamines are released, which lead to vasodilation and increase in blood vessel permeability. This is closely followed by neutrophil invasion, a process whereby neutrophils stick to the capillary endothelium walls and transmigrate across the endothelial monolayer [10]. Neutrophils have surface receptors capable of detecting bactericidal activities and they principally engulf and destroy the invading pathogen by production of oxygen free radicals, hydrogen peroxide and lysosomal enzymes. They also have high motility due to rapid amoeboid movement and respond to a wide variety of chemotactic compounds [11].

Shortly afterwards, monocytes which rapidly differentiate into macrophages are recruited to the site of infection. Macrophages are responsible for numerous homeostatic, immunological and inflammatory processes. They are distributed widely in tissue and so are well suited to provide an immediate response to invading pathogens. Activated macrophages and vascular endothelial cells release inflammatory cytokines such as $\text{TNF-}\alpha$ and IL-1. These cytokines act locally inducing further $\text{TNF-}\alpha$ production and the synthesis and secretion of other interleukins. They also have systemic effects and lead to the production of acute-phase proteins. Furthermore, the binding of $\text{TNF-}\alpha$ and IL-1 to receptors on endothelial cells, triggers the up-regulation of adhesion molecules, necessary to maintain the inflammatory response [12]. Figure 1.1 summaries the processes that mediate acute inflammation.

Additional cells mediate inflammation and sense pathogens, but are also targets of inflammatory mediators. Granulocytes are so called because they have densely stained granules in their cytoplasm that contain sacks of degradative enzymes and antimicrobial substances that digest microorganisms. There are three types of granulocytes: neutrophils, eosinophils, and basophils, each of which respond differently to signals received from sites of infection and inflammation [2].

Eosinophils are important in the defence against parasites and some fungi. They have phagocytic ability and contain catalytic enzymes in their granules, such as major basic protein and eosinophil cationic protein, which functions in parasite killing and inducing histamine release from mast cells. They have also been implicated in allergic inflammatory reactions, in which their effects can be damaging rather than protective [2]. Basophils are rare circulating granulocytes that contain many cytoplasmic granules containing histamine, proteases and powerful inflammatory mediators. They produce cytokines such as TNF- α , IL-1, IL-6, CXCL8 and IFNs, and are recruited to sites in specific inflammatory reactions, such as allergy. Their involvement in acute inflammation involves the release of histamine which acts as a vasodilator and increases vascular permeability [2]. Mast cells can be found scattered throughout the connective tissues in perivascular sites and are responsible for mediating inflammatory responses, such as hypersensitivity and allergic reactions. Mast cells store a number of different chemical mediators, including histamine, interleukins and various enzymes, in coarse granules found throughout the cytoplasm of the cell. Their activation can also lead to the synthesis of chemokines and cytokines [2].

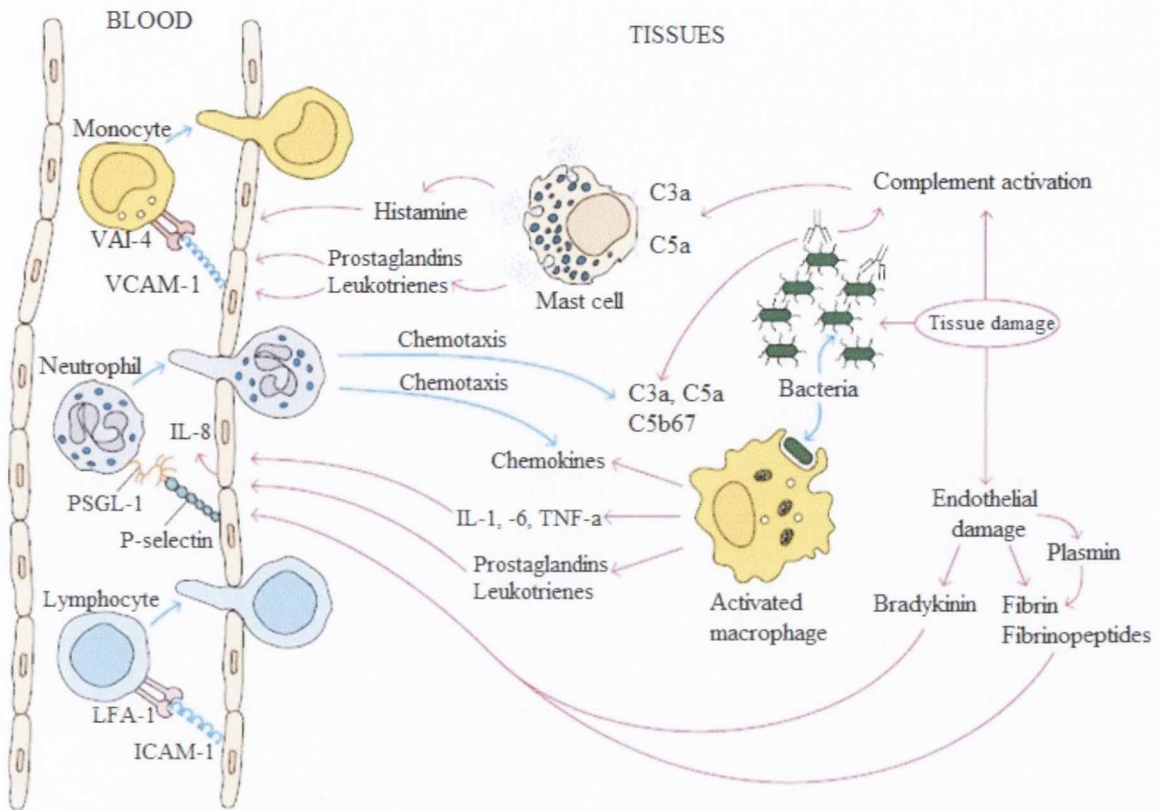


Figure 1.1: Local events during acute inflammation

Vasodilation is one of the first responses to acute injury and results in increased blood flow to the damaged area. The binding of histamines, produced by mast cells, triggers the up-regulation of adhesion molecules (Intercellular Adhesion Molecule 1 (ICAM)-1, vascular cell adhesion molecule 1 (VCAM)-1, P-selectin) which bind to corresponding receptors (lymphocyte function-associated antigen 1 (LFA)-1, P-selectin glycoprotein ligand-1 (PSGL)-1) on the surface of leukocytes. Endothelial cells lining the venules contract, widening the intercellular junctions to produce gaps which allow leukocytes (primarily neutrophils) to pass through. Neutrophils are attracted along a chemotactic gradient to the site of infection where they engulf pathogenic material. Activated macrophages release inflammatory cytokines, such as TNF- α and IL-1, which leads to an increased expression of adhesion molecules and maintains the inflammatory response. Molecular mediators such as bradykinin, prostaglandins and leukotrienes augment the immune response by contributing to vasoconstriction and smooth muscle contraction [13]. Image adapted from [14].

Inflammation is characterised by the release of pro-inflammatory cytokines such as TNF- α , IL-6 and IFN- γ and resolved by anti-inflammatory cytokines, including IL-4, IL-10 and IFN- α [15]. The outcome of the action of these pro-inflammatory cytokines is the activation of transcription factors, such as nuclear factor κ B (NF- κ B) and STATs, which ultimately initiates signal transduction pathways. The negative regulation of cytokine signalling, and the pathways they activate, is important for inflammation to ensure an effective, balanced response that limits pathology. The importance of this balance is evident in certain inflammatory diseases and chronic infections, where the inflammatory response causes more damage to the host than the infecting agent [16].

1.2.1 Triggers of inflammation

The main trigger of inflammation is the detection of microbes by receptors of the innate immune system known as PRRs, present on the surface of all cells. PRRs recognise specific repetitive structures known as pathogen-associated molecular patterns (PAMPs) of pathogens. The interaction between PRRs and PAMPs rapidly promotes immune responses such as ingestion, induction of effector molecules, like AMPs, and chemotaxis. Furthermore, their activation leads to the initiation of signalling cascades that result in the up-regulation of NF- κ B and IFN-regulatory factor (IRF) transcription factors [17]. PRRs include nod-like receptors (NLRs), retinoic-acid-inducible protein 1 (RIG-I)-like receptors (RLRs), TLRs and the intracellular DNA sensors, DNA-dependent activator of IFN-regulatory factor (DAI), IFN-inducible protein 16 (IFI16) and IFN-inducible absent in melanoma 2 (AIM2) protein.

NLRs are intracellular receptors that detect microbial components in the cytosol, such as bacterial RNA, uric acid crystals and flagellin. The NLR family is composed of 20 members each containing a C-terminal leucine-rich repeat (LRR) domain which

mediates ligand recognition, a central nucleotide-binding NACHT domain which mediates dimerization, and an N-terminal protein-protein-interaction domain composed of a caspase activation and recruitment domain (CARD) and a pyrin domain [18]. NOD1 and NOD2 are the best characterised genes out of this family. NOD1 detects meso-DAP-containing peptidoglycan found in the cell wall of gram-negative bacteria, whereas NOD2 is activated by muramyl dipeptide, which is common to both gram-negative and gram-positive bacteria [19]. Activation of NOD1 and NOD2 stimulates NF- κ B and induces the production of pro-inflammatory cytokines [20]. Other members of the NLR family (NLR family apoptosis inhibitory protein (NAIP) and NACHT-LRR-pyrin domain (PYD)-containing protein (NALP)) trigger the activation of the inflammasome, a multiprotein complex involved in activating caspase-1, which is responsible for processing pro-IL-1 into its mature active form. The inflammasome is formed by the intracellular adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the sensor protein NALP1, together with caspase-1 and caspase-5 [21].

RLRs including RIG-I, melanoma differentiation-associated antigen 5 (Mda5), and laboratory of genetics and physiology 2 (LGP2) are a family of cytoplasmic RNA helicases that recognize viral RNA and are essential for host antiviral responses [22]. RIG-I and Mda5 contain two repeats of the CARD-like motif and a DExD/H box RNA helicase domain at their N terminus, which allows them to interact with viral RNA and stimulate signalling, leading to the transcription of type I IFNs and pro-inflammatory cytokines through transcription factors IRF3 and IRF7, and NF- κ B, respectively [23,24]. RIG-I participates in the recognition of Paramyxoviruses (Newcastle disease virus), Rhabdoviruses (vesicular stomatitis virus), Flaviviruses (HCV) and

Orthomyxoviruses (Influenza), whereas Mda-5 is important for the recognition of Picornaviruses (encephalo-myocarditis virus) [23].

The Toll protein was first identified in *Drosophila melanogaster* as a membrane receptor responsible for dorso-ventral polarity, with a cytosolic domain homologous in sequence to IL-1R1 [25]. As previously mentioned, IL-1 is a potent pro-inflammatory cytokine, induced by many infectious agents and capable of inducing inflammation in all tissues [26]. Despite the similarity in sequence, Toll had no apparent function in inflammation but was known to regulate a transcription factor called, Dorsal, via a proteins kinase termed Pelle [27]. It was then discovered that Dorsal is a member of the NF- κ B family and Pelle is homologous to IL-1 receptor-associated kinase (IRAK), which revealed that a similar pathway was being used to change gene expression. In 1994, researchers into host defence in plants described N protein, which confers resistance to tobacco mosaic virus in tobacco, as homologous to IL-1R1 and Toll [28]. This domain was coined the Toll/interleukin 1 receptor (TIR) domain [29].

In 1995, a role for Toll in *Drosophila* disease resistance was uncovered, which then spurred a search for human Toll-like receptors [30]. In 1997 the first TLR was identified, and shown to activate NF- κ B, followed by four more TLRs in 1998 [31]. The mammalian TLR family now consists of 10 members (TLR1-TLR10) in humans and are characterised by the presence of LRRs in their extracellular domain and a globular cytoplasmic domain called the TIR domain. TLRs have the ability to recognise constituents of microbial cell walls or pathogen-specific nucleic acids, and engagement of TLRs triggers anti-microbial responses and cytokine production. TLR signalling proceeds via two pathways: the myeloid differentiation factor 88 (MyD88)-mediated pathway, causing activation of the transcription factor NF- κ B which induces various genes contributing to inflammatory reactions, and the TIR-domain-containing adaptor

inducing IFN- β (TRIF)-mediated pathway causing the induction of IFNs, whose stimulation leads cells to the establishment of an antiviral state [32].

Four TLR family members, TLR3, TLR7, TLR8 and TLR9, sense nucleic acid-based ligands and are localised to intracellular compartments, such as endosomes and the endoplasmic reticulum (ER). Following recognition of nucleic acids, a signalling cascade via MyD88 induces type I IFNs and inflammatory cytokines which play a critical role in eliminating viruses [23]. Double stranded (ds) RNA is produced by most viruses during replication and is the ligand for TLR3. TLR7/8 recognises single stranded (ss) RNA viruses such as influenza virus and results in the upregulation of co-stimulatory molecules and cytokine production. TLR1, TLR2, TLR4, TLR5 and TLR6 are localised on the cell surface and recognise bacterial cell wall components, resulting in the production of TNF- α and IFN- β . TLR4 is well known to recognise lipopolysaccharide (LPS) on the surface of bacteria, such as *Salmonella*, and initiate NF- κ B signal transduction leading to the expression of pro-inflammatory cytokines (Figure 1.2) [12].

Intracellular DNA sensors that induce IFNs and cytokines, in response to DNA viruses include DAI, IFI16 and AIM2. DAI is a sensor recently implicated in the recognition of foreign DNA in the cytosol [33]. This DNA sensing pathway reportedly leads to the induction of type I IFNs, and an antiviral state in the cell, via the activation of IRF3 and possibly IRF7. Loss of function studies have also indicated that DAI-mediated signalling leads to the activation of NF- κ B and thus induction of pro-inflammatory cytokines [33]. A role for DAI in the induction of IFN- β during herpes simplex virus type 1 (HSV-1) infection has been suggested, although additional DNA sensors in the cytosol may also be involved [34].

IFI16 and AIM2 form a new family of DNA receptors termed AIM2-like receptors (ALRs). IFI16 is a newly identified innate immune sensor for intracellular DNA, which mediates the induction of IFN- β [35]. IFI16 is a pyrin domain and hematopoietic interferon-inducible nuclear antigen (PYHIN) protein, containing a pyrin domain and two DNA-binding HIN domains. Experiments revealed that IFI16 bound to IFN- β -stimulating viral DNA, via the downstream component stimulator of IFN gene (STING), when stimulated with transfected DNA [35].

AIM2 detects foreign cytoplasmic dsDNA through its C-terminal HIN200 domain, leading to the oligomerisation of the inflammasome complex. This activation subsequently leads to the recruitment of ASC via the N-terminal pyrin domain. ASC also recruits pro-caspase 1 to the complex which results in the auto-cleavage and activation of caspase 1, which cleaves pro-IL-1 β , and NF- κ B. AIM2 deletion inhibits caspase-1 activation in response to cytoplasmic dsDNA and vaccinia virus [35-37].

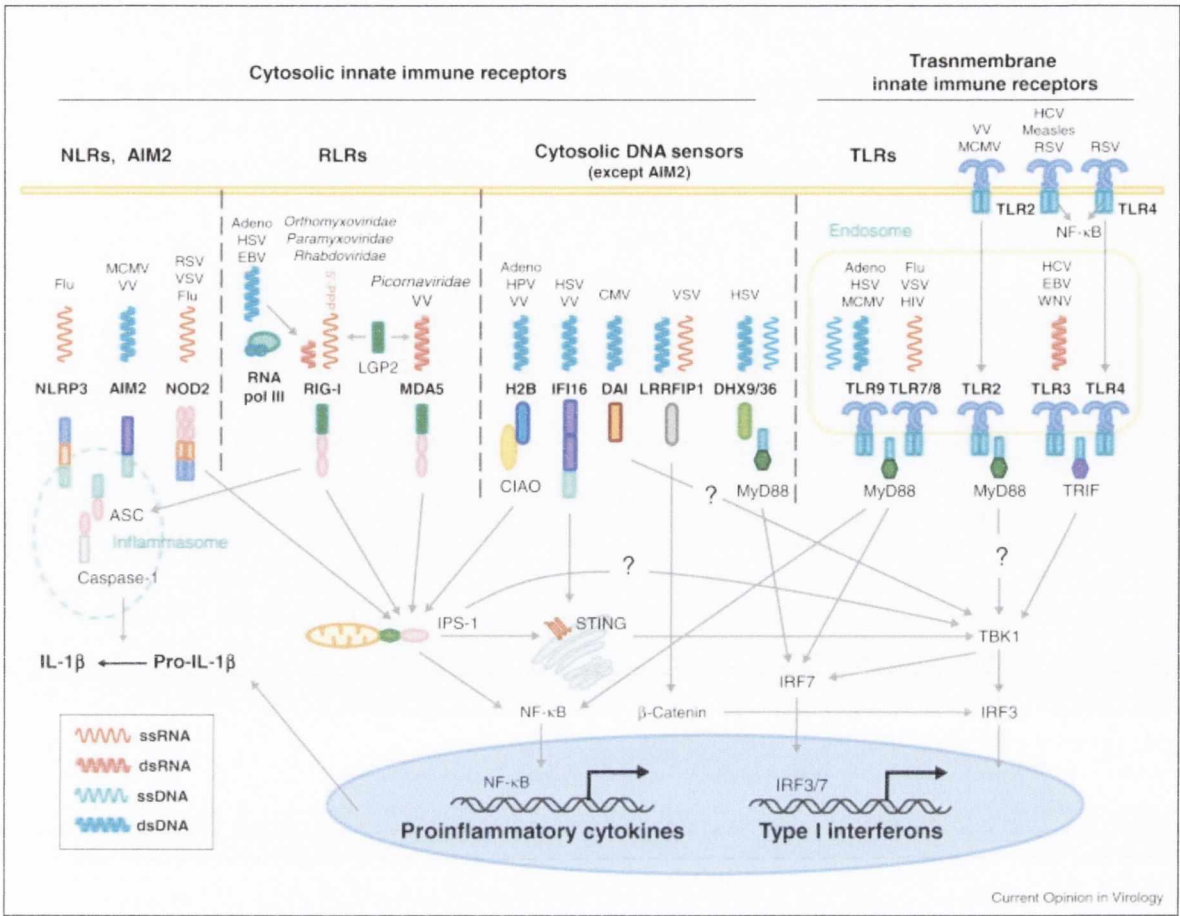


Figure 1.2: Pattern recognition receptors of the innate immune system

A schematic representation of the PRR families within the innate immune system. TLRs are membrane-bound receptors, whereas NODs, NAIPS, NALPS, as well as the nucleic acid sensors RIG-I, Mda-5, and DAI are located in the cytosol. These PRRs interact with specific adaptor molecules culminating in transcription factor activation and the induction of pro-inflammatory genes. Members of the NALP and NAIP families control activation of the inflammasome, a multiprotein complex responsible for the processing and secretion of IL-1. Image taken from [36].

1.2.2 Pro-inflammatory cytokine signalling

A key outcome of PRR signalling is the induction of pro-inflammatory cytokines and chemokines which play a major role in co-ordinating the inflammatory response. They are rapidly synthesised and induce the production of additional inflammatory mediators responsible for amplifying, prolonging and terminating inflammation.

IL-1 was defined in the 1980s as being distinctly involved in inflammation and immunity, with the capacity to affect the majority of cell types and induce inflammation [38]. IL-1 (IL-1 α , IL-1 β) is induced by multiple infectious agents and has been shown to be elevated in autoimmune diseases. The IL-1R subfamily is characterised by the presence of an extracellular immunoglobulin-like domain (Ig) and an intracellular TIR domain. IL-1R contains three Ig domains and together with the IL-1 accessory protein (IL-1RAcP) forms a receptor complex for IL-1 α and IL-1 β and the IL-1R antagonist (IL-1Ra) [39]. Receptor dimerization of IL-1R and IL-RAcP initiates a signalling cascade through the recruitment of MyD88, which results in the up-regulation of pro-inflammatory cytokines [40]. IL-1 is synthesised via the inflammasome and is responsible for the initiation of cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS), which in turn causes IL-1 exposed cells to produce prostaglandin-E2 (PGE2), platelet activating factor and NO during inflammation [41]. Furthermore IL-1 increases the expression of adhesion molecules which promotes the infiltration of inflammatory cells [41]. IL-1 can also induce fever by stimulating the release of prostaglandins, which act on the temperature control centre of the hypothalamus [6].

TNF- α was initially described as a factor that could induce necrosis in tumours. It is now known that TNF- α is a key pro-inflammatory cytokine, upregulated in response to

bacterial and viral stimuli, that has multiple significant roles in the inflammatory response, including helping to contain infection. This has been illustrated by experiments involving rabbits which were given a local bacterial infection, followed by an injection of anti-TNF- α antibody, which blocked the action of TNF- α causing the infection to spread through the bloodstream. TNF- α is predominantly produced by activated macrophages and T lymphocytes, however mast cells, neutrophils and endothelial cells can also produce it [42]. During the initial inflammatory response, TNF- α activates vascular endothelium and increases permeability which leads to the increased entry of IgG and cells into tissue. Furthermore it can increase fluid drainage to lymph nodes. The systemic effects associated with TNF- α production are fever and shock [12]. Its pro-inflammatory properties also play a crucial role in innate and adaptive immunity, cell proliferation, and apoptotic processes. TNF- α can turn on the acute phase response in hepatocytes which involves a shift in the proteins synthesised by the liver into the plasma. The acute phase proteins act as opsonins, enabling macrophages and neutrophils to ingest and destroy the invading pathogen. Excess TNF- α production as a result of deregulation can lead to many disease conditions, such as rheumatoid arthritis (RA), septic shock and inflammatory bowel disease (IBD) and anti-TNF- α blocking agents have been demonstrated to treat these conditions. In septic shock, TNF- α triggers blood clotting which results in the generation of clots in small vessels which can frequently lead to vital organ failure. Additionally, TNF- α is one of the cytokines to be produced within the inflamed joints of RA patients, leading to erosion of adjacent cartilage and bone due to the accumulation of T-cells and macrophages [12,42].

TNF- α signals through two distinct necessary receptors known as TNF receptor (TNFR)1 and TNFR2, present on the surface of most cell types [43]. The TNF receptor

superfamily is defined by an extra-cellular domain comprised of two to six repeats of cysteine clusters, however, the receptors share little sequence similarity indicating they regulate different pathways [44]. TNFR1 and TNFR2 knockout studies have indicated that TNFR1 mediates the majority of proliferation, pro-inflammatory and apoptosis-activating pathways, whereas TNFR2 mediates tissue repair and angiogenesis [45]. These receptors form multimeric complexes upon ligand binding and signals are conveyed through an array of intracellular cytosolic proteins, including TNF Receptor-Associated Death Domain (TRADD), TNFR-associated factor-2 (TRAF2) and Fas-associated protein with DD (FADD) [46]. This in turn leads to the phosphorylation of inhibitory κ B (I κ B) proteins, promoting I κ B ubiquitination and proteasome-mediated degradation. Cytosolic I κ B proteins form inhibitory complexes with the NF- κ B family of transcription factors, and I κ B degradation allows free NF- κ B to enter the nucleus and initiate gene transcription (Figure 1.3) [45]. TNF-induced NF- κ B activation induces transcription and expression of genes encoding pro-inflammatory cytokines and chemokines, such as IL-6 and CXCL8 [47].

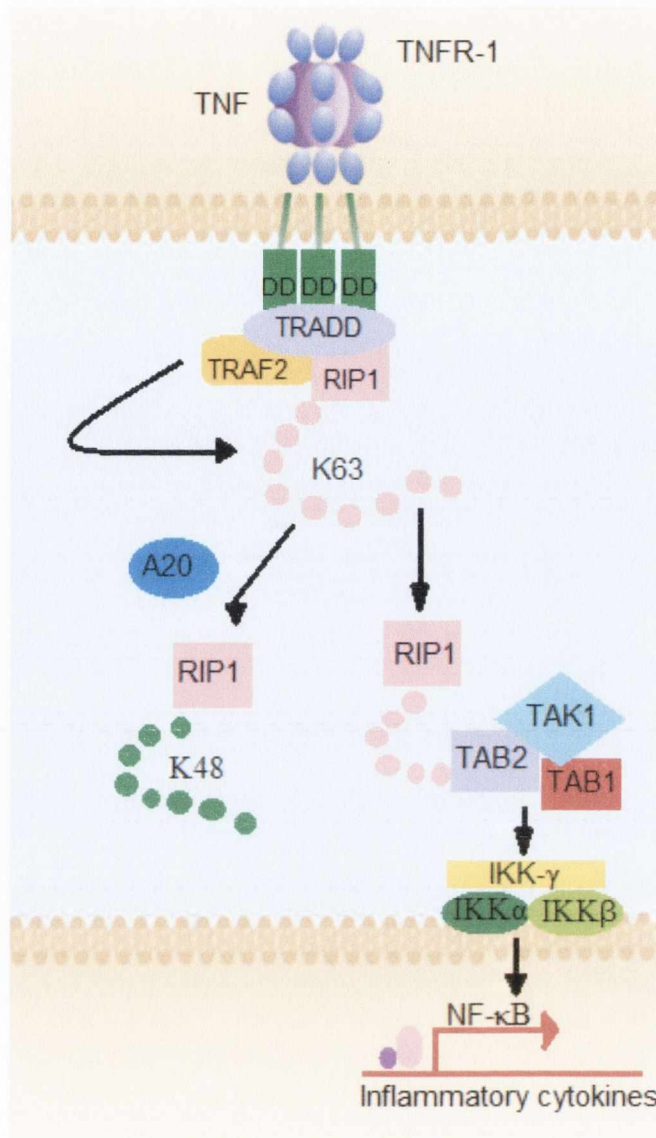


Figure 1.3: TNF- α signalling induces pro-inflammatory cytokines

In TNF- α signalling, the activated TNFR1 trimer associates with the adapter protein TRADD, which recruits receptor interacting protein (RIP)-1 and TRAF2 to the receptor complex. This in turn recruits transforming growth factor (TGF)- β activated-kinase (TAK)-1 through the binding of regulatory subunits TAK1-binding protein 1 (TAB)-1 and TAB2 to the K63-polyubiquitinated chains. TAK1 is now activated and recruits the I κ B kinase (IKK) complex. IKK phosphorylates I κ B leading to its degradation, allowing NF- κ B to translocate to the nucleus and promote transcription of pro-inflammatory genes such as IL-6, CXCL8 and TNF- α . A20 coordinates both the removal of K63-linked ubiquitin chains (pink) from RIP1 and the polyubiquitination of RIP1 with K48-linked ubiquitin chains (green), thereby targeting RIP for degradation [48]. Image adapted from [49].

IL-6 is an important cytokine with multiple roles in the regulation of inflammation, haematopoiesis, liver and neuronal regeneration, embryonal development and fertility. It has a dual effect in that it acts as both a pro-inflammatory and anti-inflammatory cytokine [50]. Synthesis of IL-6 is triggered by IL-1 and TNF- α as well as bacterial products and viral infection [51]. IL-6 initially binds to the non-signalling plasma membrane α -receptor, IL-6R α , which then recruits the common signalling receptor chain glycoprotein (gp)-130, leading to the activation of JAK-STAT and mitogen activated protein kinase (MAPK) signalling pathways. The structure of IL-6 consists of four long α -helices and the receptors comprise of fibronectin type III-like and Ig-like domains, and a cytokine binding module [50]. IL-6 is produced by a range of cell types including fibroblasts, endothelial cells and T cells, but the primary sources of IL-6 are monocytes and macrophages [52,53]. It is frequently detectable at sites of inflammation and plays a key role in the acute phase response, characterised by the production of acute phase proteins [54]. Additional functions have been attributed to IL-6, including its importance in the transition from acute to chronic inflammation, its ability to stimulate IL-1 receptor agonist which acts as an anti-inflammatory mediator and in the development of specific cellular and humoral immune responses, including end-stage B cell differentiation, Ig secretion and T cell activation [54]. IL-6 is also referred to as an endogenous pyrogen as it induces fever during an inflammatory response [12].

IL-6 plays a homeostatic role in the inflammatory response; hence, deregulated IL-6 can lead to a number of chronic inflammatory diseases including RA, IBD, osteoporosis and multiple sclerosis (MS) [50]. *In vivo* studies have established a role for IL-6 in the destruction of joints, leukocyte infiltration, apoptosis and T-cell activation during RA, furthermore, anti-IL-6 antibodies are used in the treatment of RA and Crohn's disease

[55]. Additionally, T-cells from the gastric tissue of Crohn's disease patients are resistant to apoptosis and display elevated amounts of IL-6, which activates the JAK-STAT signalling pathway, however, treatment with an IL-6R antibody induces apoptosis [56].

IFN- γ is a soluble cytokine and the only member of the type II IFN family. It signals through transmembrane glycoprotein type II receptors, IFN- γ -receptor (IFNGR)-1 and IFNGR2, whose extra-cellular domain serves as a binding site for IFN and intracellular domain associates with members of the JAK family of kinases. IFN- γ primarily utilises STAT1 which binds to IFN- γ -activated site (GAS) elements in the nucleus of cells and affects gene expression, which promotes processes such as cell cycle arrest and apoptosis [57]. IFN- γ is produced predominantly by natural killer (NK) cells, Th1 CD4 and CD8 cytotoxic T lymphocyte (CTL) effector cells and is important owing to its immunostimulatory and immunomodulatory effects, and ability to inhibit viral replication [58]. During inflammation, macrophage stimulation by IFN- γ induces antimicrobial and antitumor mechanisms, while also up-regulating antigen processing and presentation pathways. IFN- γ coordinates leukocyte attraction and directs growth, maturation and differentiation of many cell types, as well as enhancing NK cell activity and regulating B cell functions. IFN- γ is implicated in the pathology of autoimmune diseases such as systemic lupus erythematosus (SLE), MS and diabetes mellitus [59].

Chemokines are members of a family of chemoattractant cytokines, also produced in the early phase of infection. These small molecules induce chemotaxis in nearby responsive cells and CXCL8, also termed IL-8, was the first chemokine to be characterised. Chemokine receptors are seven-span membrane proteins that signal through coupled G proteins. CXCL8 and CXCL10 have two equivalent cysteine

residues separated by a single amino acid, hence the CXC motif, and bind to CXC receptors (CXCR1-6) [12].

CXCL8, previously known as neutrophil chemotactic factor, is involved in the local inflammatory reaction and is responsible for inducing neutrophils, basophils and T-cells to leave the bloodstream and migrate to the site of infection. CXCL8 acts on leukocytes as they roll along endothelial cells by initiating a change of conformation in adhesion molecules, which converts rolling to binding. It also creates a chemokine gradient to direct leukocyte migration to the surface of endothelial cells and to the site of infection [12]. CXCL8 is produced by many cell types, including mononuclear phagocytes, antigen-activated T cells, endothelial and epithelial cells [60]. Neutrophils exposed to CXCL8 and TNF- α are triggered to produce a respiratory burst that generates oxygen radicals and nitric oxide (a potent endogenous vasodilator), and to release their stored lysosomal contents, contributing to host defence and tissue destruction. CXCL8 signals through receptors CXCR1 and CXCR2, expressed upon infection by epithelial cells, and CXCR1 is essential for the increased neutrophil migration across infected cell layers *in vitro* [61]. Deregulated CXCL8 expression can lead to inflammatory diseases such as RA, IBD and psoriasis which are characterised by the accumulation of neutrophils at the site of infection and increased CXCL8 production [62].

CXCL10 is an IFN- γ -inducible chemokine that induces chemotaxis, apoptosis and cell growth inhibition during inflammation. IFN- γ is primarily produced by activated T cells which induce different cells types to produce CXCL10. This enables CXCL10 in turn to attract and recruit Th1 cells, suggesting the existence of a positive feedback loop between IFN- γ producing Th1 cells and resident cells producing CXCL10 [63]. Interestingly, a recent study in the human acute monocytic leukaemia cell line, THP-1, revealed that TNF- α induces CXCL10 with a greater potential than IFN- γ [64].

Furthermore, IFN- γ enhanced the TNF- α -induced production of CXCL10 by activating STAT1 and NF- κ B, respectively [64].

CXCL10 is produced by keratinocytes, monocytes, activated T cells and fibroblasts and elicits its effects by binding to the cell surface receptor, CXCR3, and attracting NK cells, CD4⁺ Th1 cells, CD8⁺ T cells and monocytes to inflamed sites [12]. Binding of CXCL10 with its receptor CXCR3, results in increased chemotactic activity through the p38/MAPK and phosphoinositide-3-kinase (PI3K) signalling pathways [63]. Moreover, it has been reported to be involved in the regulation of lymphocyte chemotaxis and in the inhibition of tumour growth, by acting as an angiostatic agent [65]. Previous studies have found that CXCL10 expression is increased in inflammatory disorders such as RA, SLE and MS. CXCL10, mainly produced by infiltrating macrophage-like cells, has been detected in the sera, synovial fluid and synovial tissue in RA patients [66].

In addition to the up-regulation of pro-inflammatory cytokines and chemokines, stimulated PRRs also induce IFN-regulatory factors which in turn activate a very important family of proteins, known as type I IFNs. These proteins play a significant role in the regulation of innate and adaptive immunity, cell growth and proliferation, and contribute to the body's antiviral defence system.

1.2.3 Type I IFNs

Stimulation via TLR3, 7, 8, 9 and RIG-I results in the production of multiple subtypes of thirteen human α -IFNs and a single β -IFN belonging to the type I IFN family. IFNs were initially discovered, by Isaacs and Lindenmann in 1957, as soluble proteins that inhibited viral replication in cell culture [67]. To date, IFNs are recognised as not only prominent antiviral effectors, but essential pleiotropic cytokines that have anti-proliferative, anti-angiogenic and immunomodulatory effects [68]. IFNs are so called

because they interfere with virus replication and are responsible for the first line of defence during viral infection; however, they also play an important part in immune surveillance for malignant cells [69]. Genetically engineered mice lacking responsiveness to IFN- α/β rapidly succumb to viral infections; furthermore, humans with defects in STAT1, which is a component of the IFN signalling cascade, die of viral diseases at an early age [70,71].

ds- and ssRNA, produced during viral infection, activates specific PRRs which mediate a signal to CARD adaptor inducing IFN- β (Cardif), also commonly known as MAVS, IPS-1 or VISA [72]. Cardif activates two IKK-related kinases, IKK ϵ and serine/threonine-protein kinase (TBK)-1, which phosphorylate the transcription factor IRF3 [73]. IRF3 phosphorylates, homodimerises and translocates to the nucleus to initiate IFN- β mRNA synthesis [74]. IRF7 is activated in a similar way as IRF3 resulting in a positive feedback loop that leads to the production of IFN- α .

IFNs are secreted into the surrounding medium and act on uninfected neighbouring cells to activate a global antiviral state. IFNs can directly exert their effect via the IFN- α receptor (IFNAR) or indirectly via the induction of chemokines, which leads to the recruitment of immune cells to the site of infection, secretion of cytokines or by stimulating other cell types [75]. Fibroblasts mainly secrete IFN- β while dendritic cells (DCs) are the primary producers of IFN- α [76]. Secreted IFN- α signals to cells to induce the expression of hundreds of genes, known as IFN-stimulated genes (ISGs), that activate antiviral and biological processes, including amplification of IFN signalling, production of cytokines that activate adaptive immunity and many factors that directly inhibit viruses [77]. Some important biological activities induced by type I IFNs include the modulation of the expression of major histocompatibility complex

(MHC)-I, the stimulation of CD8+ and NK cell activity and the activation of signalling pathways such as MAPK and NF-κB [75,78].

IFNs were the first cytokines that proved to be effective in the treatment of malignancies [57]. The use of type I IFN for therapy is widespread, with IFN being administered for the treatment of a variety of diseases including solid and hematologic malignancies, MS and most importantly chronic viral hepatitis [75]. Type I IFNs can inhibit the replication of normal and tumour cells by prolonging G1 and reducing the rate of entry into S-phase [78]. Furthermore, they can increase MHC class I expression which enhances antigen recognition, represses oncogene expression and induces tumour suppressor genes [75]. FDA approval has been received for the use of IFN-α in the following six diseases (Table 1.2), and its use in combination with other cytokines in order to improve the efficacy of therapies, is under current review [57].

Indication	Role of IFN	Ref.
Hairy cell leukemia	Promote remission	[79]
Chronic myelogenous leukemia (CML)	Induces T-cell mediated responses against CML	[80]
Cutaneous T-cell lymphomas	Active agent in treatment of mycosis fungoides	[81]
Multiple Myeloma	Cells responsive to anti-proliferative effects	[82]
Melanoma	Prolongs relapse free survival	[83]
Viral Hepatitis	Induces antiviral ISGs	[84]

Table 1.2 IFN-α therapy approved diseases

1.2.4 Type I IFN signalling: JAK-STAT pathway

The JAK-STAT signalling pathway was the first to be recognised as eliciting the biological effects associated with the IFNs [85]. Secreted IFNs signal through the IFNR complex which is formed by IFNR1 and IFNR2, resulting in receptor subunit dimerisation and tyrosine phosphorylation of the receptor proteins. The tyrosine kinases involved in this process are members of the JAK family (JAK1-3 and tyrosine kinase (TYK) 2). Activated JAKs phosphorylate the receptor subunits and specific latent cytoplasmic transcription factors called STATs (STAT1-4, 5a, 5b and 6) [86].

Type I IFNs usually transduce their signals to STAT1 and STAT2, which form a heterodimer and associate with IRF-9, forming a complex known as IFN-stimulated gene factor (ISGF)-3. ISGF3 migrates to the nucleus and binds to the promoter region of IFN-sensitive response elements (ISRE), inducing the transcription of more than 500 ISGs that have antiviral, immunoproliferative and immunomodulatory functions (Figure 1.4) [85]. Many of these ISGs have no known function, however, some of the best characterised antiviral ISGs are summarised below (Table 1.3). Collectively, ISGs have been shown to inhibit every stage of viral replication, however, viruses such as HCV have evolved efficient strategies to counteract this IFN- α -induced immune response [87]. Additional roles for ISGs in biological processes include apoptosis, immune modulation, inflammation, development and signalling [88].

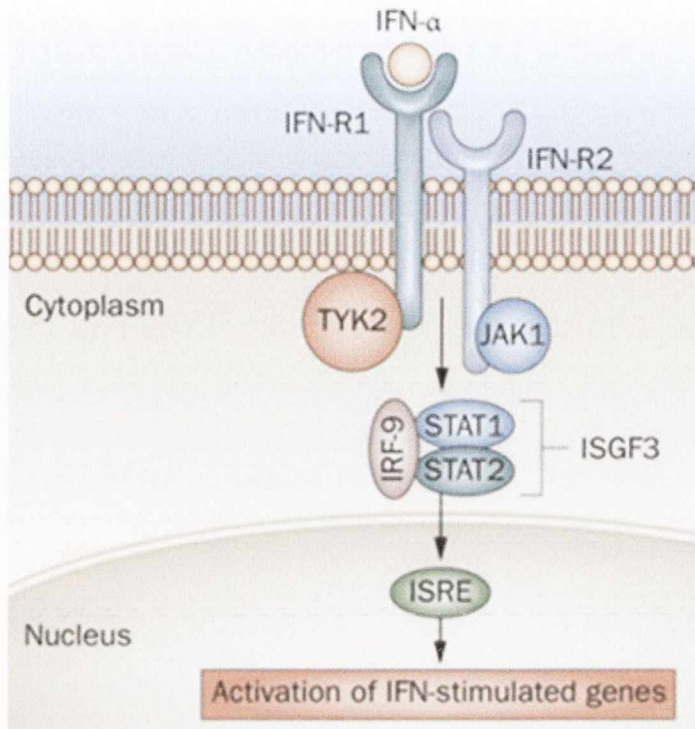


Figure 1.4: Overview of type I IFN signalling

By interacting with specific receptors, IFN- α activates STAT proteins which initiate the JAK-STAT signalling cascade. JAKs then associate with the receptor and phosphorylate STATs. ISGF3 complex forms, which contains a third transcription factor, IRF-9, and translocates to the nucleus. The ISGF3 complex binds to a specific nucleotide sequence known as the ISRE leading to the up-regulation of specific ISGs including Protein kinase R (PKR), Myxovirus resistance A (MxA) and 2'-5' oligoadenylate synthetase (OAS). Image taken from [89].

ISG	Virus Inhibited	Mechanism of action	Ref.
MxA	Orthomyxoviruses, Paramyxoviruses, Rhabdoviruses, Togaviruses, Bunyaviruses, Influenza, Coxackie virus	Dynamin-like GTPase that inhibits viral multiplication by binding and sequestering the nucleocapsid protein into large perinuclear complexes May also form oligomeric rings around tubular nucleocapsid structures, thereby inhibiting their transcriptional and replicative function	[90]
PKR	Encephalomyocarditis virus, Vaccinia, Human immunodeficiency virus (HIV)-1	PKR is a serine-threonine kinase that phosphorylates and inactivates the cellular translation factor eIF-2a upon binding dsRNA, consequently blocking the translation of cellular and viral mRNAs	[91]
2'-5'OAS	ssRNA viruses, Picornaviridae, Reoviridae, Togaviridae, Paramyxoviridae, Orthomyxoviridae, Flaviviridae	Possesses dsRNA-regulated nucleotidyl transferase activity, oligomerizing ATP into 2'-5' linked oligoadenylates, which activate a latent endoribonuclease, RNaseL, that degrades viral RNA	[92]
Viperin (Cig5)	Human cytomegalovirus, HCV, Influenza, HIV- 1	ER-associated ISG, disrupts cell plasma membrane and lipid rate integrity, inhibits influenza virion budding	[93]
ISG15	Influenza, Sinbis, HSV1, MHV68	Ubiquitin-like protein that modifies ubiquitination on cellular and viral targets, inhibits viral replication, ISGylation inhibits viral budding of Ebola	[94]
IFITM (IFN- inducible transmembrane) 1,2,3	Influenza, Dengue, West Nile virus, VSV	Inhibits viral entry	[95]

Table 1.3: Summary of the function of known antiviral ISGs

1.2.5 Resolution of inflammation

The normal resolution of inflammation is the replacement of damaged with organised tissue, identical in structure and function to the original. Together, new blood vessels are generated at the periphery of the injury and fibroblasts secrete collagen to form granulation tissue. Eventually the blood vessels regress and all that remains is collagen which becomes tougher over time [96]. Furthermore, macrophages and lymphatics mediate the removal of oedema and plasma proteins resulting in normal vascular permeability. However, when the regulatory mechanisms of inflammation are defective, prolonged active inflammation with concurrent healing and repair results in chronic inflammation. Increased capillary permeability causes neutrophils to continually leave the blood and accumulate at the infection site. Subsequently, surrounding tissue is destroyed and replaced with scar tissue due to discharged lysosomal contents and reactive oxygen species [97].

Dysregulated inflammation may give rise to chronic diseases such as coeliac disease, RA and IBD. In these autoimmune diseases, the constant mechanisms of destruction are being directed at the body's own tissue, due to the presence of new autoantigen. RA is classed as a T-cell mediated disease, characterised by inflammation of the synovium. Disease progression leads to inflamed synovium which invades and damages the adjacent cartilage, resulting in bone erosion and loss of function. DCs and macrophages induce pro-inflammatory cytokines which stimulate the differentiation of B cells, into arthritogenic antibody producing plasma cells. Another common inflammatory disease is an intestinal disorder known as IBD or Crohn's disease, which is caused by an abnormal over-responsiveness to normal commensal gut flora. Patients suffer from episodes of severe inflammation primarily affecting the terminal ileum. The disease is

characterised by a chronic inflammation of the mucosa and submucosa of the intestine which results in the dense infiltration of lymphocytes and development of granulomatous lesions. These autoinflammatory diseases illustrate well the importance of the precise regulation of inflammation [12].

Furthermore, inflammation is a common sequel to viral infection with effective antiviral immunity. The interaction of viruses with innate immune receptors initiates the induction of inflammatory and immunomodulatory cytokines in the body. HCV is the second most prevalent chronic infection, hepatitis B being the first, in the world. About 75% of people infected develop chronic infection, with viral persistence leading to prolonged liver inflammation and fibrosis. Between 5% and 20% of those chronically infected will go on to develop cirrhosis and steatosis, leading to hepatocellular carcinoma (HCC) [98].

1.3 Regulation of Inflammation

The inflammatory process requires strict regulation at several levels which can be achieved by endogenous mechanisms, including negative cytokine signals and ubiquitination, or therapeutically via anti-inflammatory drugs.

1.3.1 Therapeutic regulation

Immunotherapy depends on three types of drugs, including anti-inflammatory, cytotoxic and immunosuppressive, which can reduce tissue injury inflicted by an inflammatory response or reduce an autoimmune response. The most commonly used anti-inflammatory agent is aspirin, which is well appreciated for its ability to inhibit the biosynthesis of prostaglandins and generate anti-inflammatory mediators [99]. For more severe diseases, anti-inflammatories are combined with cytotoxic or immunosuppressive drugs. Anti-TNF- α antibodies are potent anti-inflammatories which

have been found to induce remission in RA and reduce tissue inflammation in Crohn's disease, by binding to the pro-inflammatory cytokine, TNF- α , and blocking its activity. Cytokines IL-1, IL-15 and IL-6 are also targeted in the treatment of RA; however, these therapies are less effective and associated with an increased risk of infection. Furthermore, treatment with anti- α_4 integrin and CD20-specific antibodies reduces the relapses of MS and improves RA by blocking the adhesive interaction between lymphocytes and monocytes on endothelial cells, thereby preventing the cells from entering the tissue and exacerbating inflammation. T-cell targeted therapies have also been shown to be effective in the treatment of RA and psoriasis, by inhibiting the activation of keratinocytes, T cells and DCs [12].

Corticosteroid drugs are powerful anti-inflammatory and immunosuppressive agents that are used to dampen the harmful effects of the immune response. They are lipid soluble molecules that diffuse across plasma membranes to enter cells and bind cytosolic receptors. Receptor binding displaces heat-shock proteins and exposes the DNA-binding region of the receptor. Steroids can act by binding to the promoter region of steroid-responsive genes or interacting with transcription factors such as NF- κ B. These drugs predominantly target the functions of monocytes and macrophages and reduce the production of inflammatory mediators, inhibit the expression of adhesion molecules and promote apoptosis of leukocytes [12].

The transplantation of tissues to replace diseased organs is an important medical therapy; however, the immune response to grafted tissue is a major impairment of successful transplantation. Rejection is caused by the immune response to alloantigens, foreign proteins on the graft tissue that vary in individuals, which triggers a response against grafted tissue/organs. To overcome this, MHC matching of donor and recipient

is attempted; though this is mostly successful when the donor and recipient are related. Immunosuppressive drugs such as cyclosporin A and tacrolimus have greatly improved graft survival by inhibiting T-cell proliferation in response to allogenic cells; however, they can be cytotoxic to kidneys and can affect all immune responses.

Antibodies are an alternative therapy to immunosuppressive drugs that are not associated with cytotoxic side effects. Anti-lymphocyte globulin is used to treat graft rejection by removing unwanted lymphocytes, but cannot discriminate between useful and unwanted lymphocytes. Campath-1H is a monoclonal antibody directed at CD25 on the surface of lymphocytes, used in solid organ and bone marrow transplantation. Furthermore, co-stimulatory molecules B7.1 and B7.2 on the surface of APCs, required to activate T-cells that recognise donor antigens, have been targeted to inhibit allograft rejection by recombinant protein CTLA-4-Ig [12].

Collectively the development of anti-inflammatory immunotherapy has been a triumph, however, it also has the disadvantage of being expensive, cumbersome to administer and associated with an increased risk of infection.

1.3.2 Anti-inflammatory cytokines

If the inflammatory response has gained control of the infection, the overall reaction is shifted towards anti-inflammatory signals. The signals that mediate this shift come from lipoxins, protectins and resolvins. Lipoxins halt the recruitment of neutrophils, promote the uptake of apoptotic neutrophils by macrophages and recruit additional monocytes to help eliminate dead cells. Macrophages, neutrophils and epithelial cells can also produce secretory leukocyte protease inhibitor (SLPI), which inactivates proteases released from neutrophil granules and pushes the inflammatory response toward resolution [100].

Anti-inflammatory cytokines including IL-4, IL-6, IL-10, IL-11, IL-13 and TGF- β are produced by Th-2 type cells and serve to counteract various aspects of inflammation, and limit the potentially damaging effects of sustained inflammation. Table 1.4 summarises their mechanism of action. Anti-inflammatory cytokines are already proven efficacious through their use in clinical conditions involving excess inflammation. IL-11 is a counter-therapy for chemotherapy induced thrombocytopenia, while IL-10 is currently in clinical trials for the treatment of RA and IBD [101].

Anti-inflammatory cytokine	Mechanism of inhibition
IL-4	Promotes Th2 lymphocyte development; inhibition of LPS induced pro-inflammatory cytokine synthesis
IL-6	Inhibition of TNF and IL-1 production by macrophages
IL-10	Inhibition of monocyte/macrophage and neutrophil cytokine production and inhibition of Th1-type lymphocyte responses
IL-11	Inhibits pro-inflammatory cytokine response by monocyte/macrophages and promotes Th2 lymphocyte response
IL-13	Shares homology with IL-4 and shares IL-4 receptor; attenuation of monocyte/macrophage function
TGF- β	Inhibition of monocyte/macrophage MHC class II expression and pro-inflammatory cytokine synthesis

Table 1.4: Anti-inflammatory cytokines’ mechanisms of action [101].

1.3.3 Ubiquitin mediated regulation

Ubiquitination of signal transduction pathways is increasingly recognised as a mechanism of regulation during inflammation. Ubiquitins are small regulatory molecules that attach to proteins and label them for degradation via the proteasome. Ubiquitination is carried out by a set of three enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ligating enzyme. The E3 ubiquitin ligase binds the specific substrate and mediates the covalent attachment of the ubiquitin molecules. Ubiquitination of the pro-inflammatory cytokine-induced NF- κ B signalling pathway is a well-established mechanism of control [102]. NF- κ B transcription factors mediate the effects of pro-inflammatory cytokines and failure to regulate NF- κ B activity results in chronic inflammation and cell death [103]. The first identified target of ubiquitination in the TNFR1 signalling cascade is I κ B which, following TNF- α stimulation, becomes ubiquitinated by the Skp1-cullin-F-box protein (SCF) ^{β TrCP} E3 ligase and degraded via the proteasome, allowing NF- κ B transcriptional activity [104]. Since its discovery, additional ubiquitin targets within the TNFR1 pathway have been discovered, such as RIP1, cellular FLICE-inhibitory protein (cFLIP), caspase-8/10 and TRAF2 [102].

A20, a critical negative regulator of the TNFR1 signalling cascade, down-regulates the pathway through the activity of two ubiquitin-editing domains [103]. Polyubiquitin chains linked by lysine (Lys)-48 residues often target substrates for proteasome mediated degradation whereas Lys (K)-63 polyubiquitin chains do not promote proteasomal degradation, instead regulate processes within the cell such as activation of pro-inflammatory transcription factors [102]. Firstly, the amino terminal domain of A20, with contains a de-ubiquitinating (DUB) enzyme, removes K63-linked ubiquitin

chains from RIP, an essential mediator of NF- κ B signalling. Secondly, the A20 E3 ligase domain promotes ligation of Lys-48-linked ubiquitin chains to RIP, thereby targeting RIP for proteasomal degradation and subsequently down-regulating the NF- κ B signalling pathway [48]. A20 presents as a dual functioning ubiquitin-editing enzyme, necessary for the regulation of TNF- α , as demonstrated by A20 knockout mice which suffered rampant inflammation in multiple organ systems as a result of deregulated NF- κ B activity [103].

Additional negative regulators of TNFR1 signalling, such as cylindromatosis tumour suppressor (CYLD) and seven in absentia homolog (SIAH) proteins have been shown to be vital in the regulation of this pathway. CYLD is a de-ubiquitinating protease that targets TRAF2 and NEMO and releases its K63-linked ubiquitin chains, thereby attenuating downstream signalling cascades. SIAH proteins promote TRAF2 Lys-48-linked ubiquitination and degradation, thereby promoting its turnover. Thus we can conclude that the process of ubiquitination controls cellular processes and signalling pathways in order to maintain homeostasis and limit inflammation related pathology [102].

1.3.4 SOCS

Runaway cytokine signals can have detrimental biological consequences, including tumour growth, inflammatory diseases and autoimmune disorders [105]. It is therefore imperative that the amplitude and longevity of the signal is tightly controlled, in order to limit tissue damage. The JAK-STAT signalling pathway is under tight regulation by a group of proteins known as SOCS. SOCS proteins are key physiological regulators of inflammatory pathways and play a vital role in maintaining homeostasis in the cell. By modulating the balance of essential signalling pathways, SOCS proteins determine the

fate, differentiation and response of cells. Most SOCS proteins are induced by a plethora of cytokines and act in a negative feedback loop to inhibit cytokine signal transduction (Table 1.5).

SOCS	Inducing factor	Signalling inhibited by SOCS
CIS	IL-2, IL-3, IL-6, IL-9, IFN- α , TNF- α , erythropoietin (EPO), growth hormone (GH), thymic stromal lymphopoietin (TSLP), prolactin	IL-2, IL-3, EPO, GH, prolactin
SOCS1	IL-2, IL-4, IL-6, IL-7, IL-9, IL-13, IFN- α/β , IFN- γ , leukemia inhibitory factor (LIF), TNF- α , EPO, TSLP, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage (GM)-CSF, M-CSF, GH, prolactin, insulin, ciliary neurotrophic factor (CNTF), cardiotropin, thyroid stimulating hormone (TSH), LPS, CpG DNA	IL-2, IL-4, IL-6, IL-7, IL-12, IL-15, IFN- α/β , IFN- γ , LIF, TNF- α , EPO, TSLP, GH, prolactin, insulin, leptin, LPS, CpG DNA
SOCS2	IL-6, IFN- α , IFN- γ , LIF, GH, prolactin, insulin, CNTF, cardiotropin	IL-6, GH, insulin-like growth factor 1 (IGF-1)
SOCS3	IL-1, IL-2, IL-4, IL-6, IL-9, IL-10, IL-13, IFN- α , IFN- γ , LIF, EPO, GM-CSF, GH, prolactin, insulin, leptin, CNTF, LPS, CpG DNA	IL-1, IL-2, IL-4, IL-6, IL-9, IL-11, IL-12, IFN- α/β , IFN- γ , LIF, EPO, GH, prolactin, insulin, leptin
SOCS4	Epidermal growth factor (EGF)	EGF
SOCS5	IL-6, IL-12, EGF	IL-4, IL-6, EGF
SOCS6	Insulin, SCF	Insulin, SCF
SOCS7	Insulin, IGF-1	Insulin

Table 1.5: Factors induced and signalling inhibited by SOCS proteins [106,107].

The SOCS family of proteins consists of eight members: cytokine-inducible Src homology 2 (SH2) domain-containing protein (CIS) and SOCS1-7 (Figure 1.5). They are cytoplasmic proteins and their structure consists of a central SH2 domain, a C-terminal domain, coined the SOCS box and a variable N-terminal region [108,109]. The SH2 domains of SOCS proteins are involved in substrate binding through recognition of

distinct phosphorylated tyrosine motifs on target proteins. SH2 domains are evolutionarily conserved and specifically function in protein tyrosine kinase (PTK) pathways, due to the dependence on tyrosine phosphorylation binding [110]. The SOCS box is 50 amino acids in length, 20% conserved among the family, and interacts with several ubiquitinating machinery enzymes to form an E3 ligase complex [111]. The N-terminal is also thought to be involved in binding target molecules.

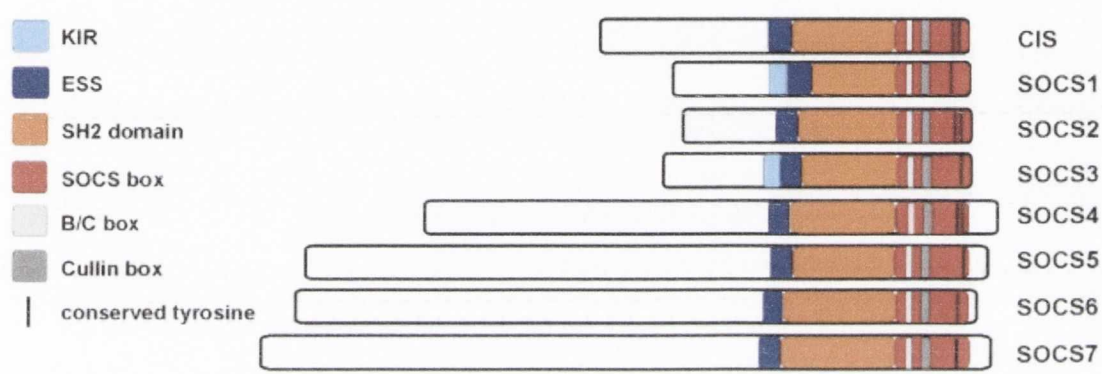


Figure 1.5: Domain organisation of SOCS family members

All SOCS proteins contain a conserved SH2 domain which is involved in binding target proteins, an N-terminal domain and a SOCS box for binding to the E3 complex. SOCS1 and SOCS3 are the only members of the family to contain a kinase inhibitory region (KIR) region which directly inhibits the tyrosine kinase activity of JAK. Image taken from [109].

1.3.5 SOCS mediated regulation

SOCS are themselves regulated via several mechanisms. The SOCS box functions as an E3 ubiquitin ligase that can target specific proteins for ubiquitin-mediated degradation [112]. SOCS proteins form Elongin C–Cullin–SOCS box (ECS)-type complexes that function as E3 ubiquitin ligases. This is achieved when Elongin B binds Elongin C, which bridges the target substrate protein recognised by the SOCS box, to a Cullin scaffold protein. Cullin then recruits a RING finger containing protein Rbx, which completes the assembly of the E3 ligase [109]. In this way, SOCS proteins can regulate the half-life of a wide range of tyrosine phosphorylated target proteins.

Much of our knowledge of SOCS proteins and their function come from studies of SOCS1 and SOCS3. There are four main ways that SOCS1 and SOCS3 inhibit JAK-STAT signalling: 1) compete with STATs for cytokine receptor binding; 2) target the receptor for degradation via the proteasome; 3) bind to JAKs and directly inhibit their kinase activity and 4) target JAKs for degradation by the proteasome [111]. Although the structure of SOCS1 and SOCS3 is similar, their mechanism of regulation requires different attachment points on the JAK-STAT pathway (Figure 1.6).

SOCS1 binds a specific phosphorylated tyrosine residue site, Y1007, on the activation loop of JAKs and residues, Y1146, Y1150 or Y1151 on the insulin receptor. SOCS3 does not have as high a binding affinity for the activation loop of JAKs but rather preferentially binds the receptor subunit. SOCS3 has been reported to bind to tyrosine residues in gp130 related cytokine receptors, such as Y759 in gp130 and Y800 in IL-12 receptor β 2, and also activated GH receptor, leptin receptor and EPO receptor [112].

SOCS knockout studies have revealed individual roles for each SOCS protein and also established them as important biological regulators of immune and inflammatory

processes. An indispensable role for SOCS1 emerged when SOCS1 knockout mice died within 3 weeks of birth from severe lymphopenia, fatty degeneration of the liver and macrophage infiltration of major organs, primarily as a result of unbridled IFN- γ signalling [113]. Although this can be rescued, with null mutations of IFN- γ , mice eventually succumb to inflammatory disease, indicating that a SOCS1 deficiency is associated both with acute and chronic inflammatory disorders [114].

SOCS3 knockout mice initially develop normally but soon show marked liver erythropoiesis with consequent embryonic death, possibly due to deregulated LIF signalling [113]. More recent studies using conditional SOCS3-deficient mice, and SOCS3-deficient macrophages, have shown that loss of SOCS3 leads to sustained IL-6 activation of STAT3, postulated to be associated with the progression of chronic inflammatory diseases such as Crohn's disease [115].

SOCS proteins also regulate other pathways such as those involved in NF- κ B and TLR signalling. The downstream TLR adaptor, Mal, is a target of SOCS1. Mal undergoes Bruton's tyrosine kinase-dependent tyrosine phosphorylation following TLR stimulation, and is thereby targeted by SOCS1-mediated degradation. Overexpression studies have shown that SOCS1 interacts with Mal, via the SH2 and SOCS box domain of SOCS1, polyubiquitinates and thus degrades it [116]. SOCS1 also negatively regulates the MyD88-dependent pathway by binding to the p65 subunit of NF- κ B. IKK α mediates phosphorylation of NF- κ B p65/RelA and SOCS1 interacts with this subunit, enhancing its ubiquitination and degradation, thereby inhibiting NF- κ B signalling [117].

Although SOCS1 directly targets TLR signalling molecules, SOCS3 acts to influence the secondary effects of LPS stimulation by altering the cytokine profile. SOCS3 is

induced by the anti-inflammatory cytokine IL-10 and has been reported to facilitate the IL-10 mediated inhibition of LPS-induced macrophage. Murine macrophages transfected with SOCS3 inhibited the production of LPS-induced NO, TNF- α , IL-6 and GM-CSF in response to LPS, when compared to wild-type (WT) cells [112]. Reports also suggest that SOCS3 mediates the downstream effects of IL-6 and IL-10 in response to LPS. Both IL-6R and IL-10R signalling involves JAK1-STAT3 signalling, however, SOCS3 can bind to the phosphorylated tyrosine residue of the IL-6R subunit, gp130, thereby inhibiting IL-6-mediated STAT3 activation, but it does not bind to IL-10R [115]. Moreover, studies have shown that SOCS3 can suppress TNF- α -induced phosphorylation of the MAPKs ERK1, ERK2, p38 and c-Jun N-terminal kinases (JNK) and repress TNF- α -induced degradation of I κ B, in primary rat islets and in a pancreatic insulin-producing rat beta cell line [118]. Negative regulation of inflammatory cytokines is important to allow the beneficial immunological effect of the cytokine, while preventing the pathological consequence of an uncontrolled one. These findings demonstrate the importance of SOCS proteins and the essential physiological role they play in maintenance of homeostasis.

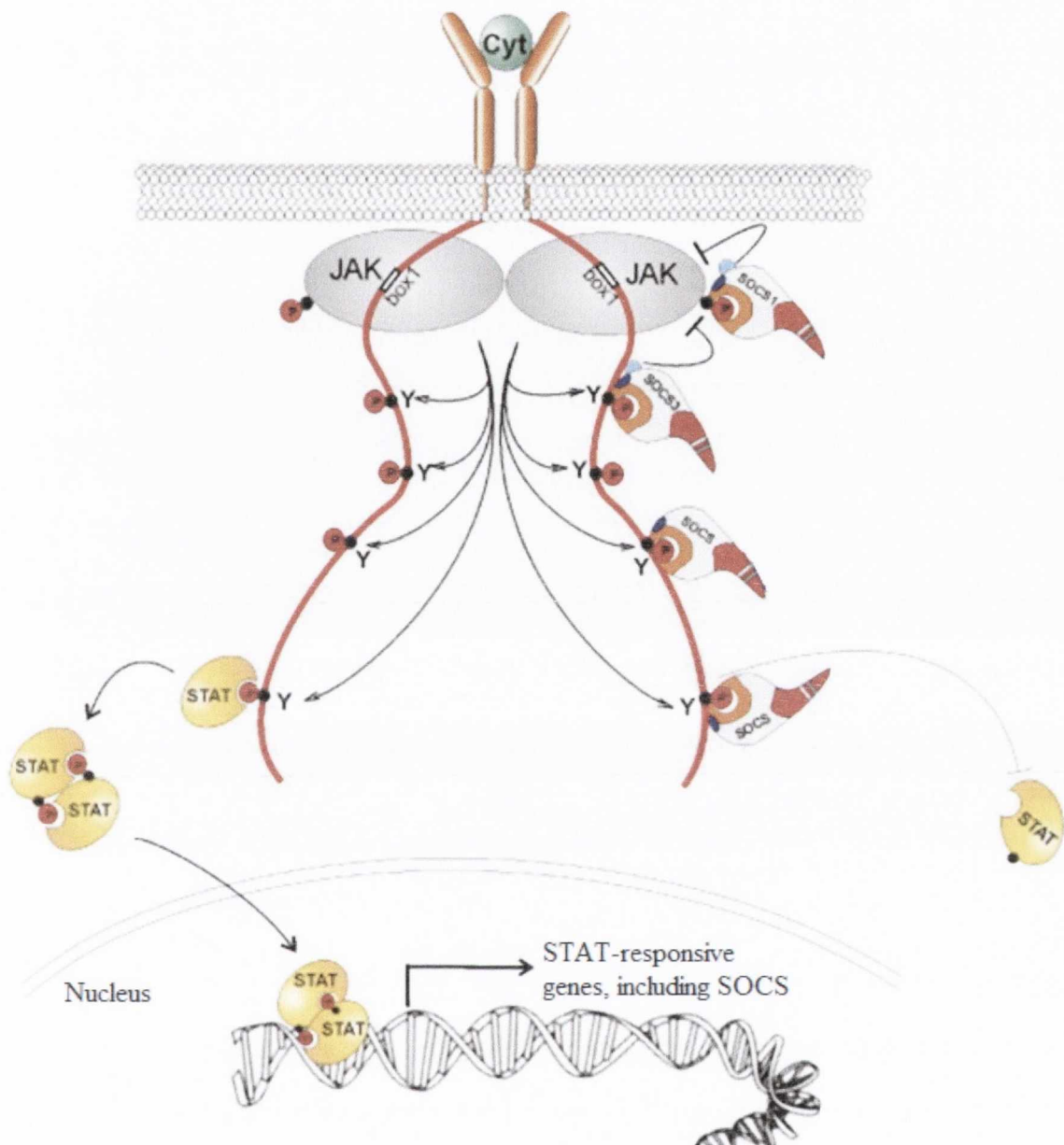


Figure 1.6: Inhibitory mechanism of SOCS

Cytokine stimulation induces SOCS via the JAK-STAT signalling pathway. The induced SOCS proteins can block the interaction of STATs with receptors, compete via SH2-domain-mediated binding to JAKs and cytokine receptors, or inhibit the catalytic activity of JAKs through binding via the KIR and SH2 region. SOCS1 and SOCS3 can form functional E3 ubiquitin ligases through the assembly of ElonginBC, Rbx, Cul5 and SOCS. Image taken from [109].

1.3.6 The regulation of SOCS

Following stimulation, SOCS1 and SOCS3 are rapidly induced and promptly degraded, indicating that their half-life is specifically controlled. In addition to their role as regulators of cytokines and signalling pathways, SOCS proteins themselves are regulated. Alternative splicing and mRNA stability are important mechanisms of SOCS regulation [111]. SOCS1 contains two start codons, one of which leads to premature termination of translation; while SOCS3 can undergo alternative mRNA initiation, which results in the exclusion of an exon encoding an ubiquitination site, thereby increasing SOCS3 stability. This truncated version of SOCS3 was found to be induced by ER stress and RNA activated PKR, as a result of the induction of eukaryotic initiation factor 2 α phosphorylation. The outcome caused stronger cytokine inhibitory activity and stability [119,120]. The presence of a PEST (Pro, glutamic acid (E), Ser and Thr) motif in the SH2 region of SOCS3 appears to mediate non-proteasomal degradation [120]. Furthermore, phosphorylation within the SOCS box results in loss of interaction with elongin B/C, which limits SOCS3 turnover [121]. SOCS molecules can also degrade other SOCS proteins, with SOCS2 reported to bind all members of the family [122]. Although the exact mechanism has yet to be uncovered, the effect is thought to rely on the presence of the SOCS box, in both the target SOCS and in SOCS2. It is suggested that SOCS2 may target other SOCS proteins for proteasomal degradation or compete with them for Elongin BC binding [109,122].

SOCS proteins have a powerful role in the negative regulation of signalling pathways to prevent excessive inflammation while maintaining protective antiviral responses. As such, they are frequently the target of viral exploitation and a number of viruses have hijacked their mechanisms of control, to promote virus survival. Additionally, their role

as regulators is as important as their own regulation, although, much less is known about the latter. Deregulated SOCS expression has been shown to contribute to chronic inflammatory diseases, viral infection and cancer. MicroRNAs (miRNAs) are recently identified negative regulators of gene expression and have been implicated in the immune response. We hypothesise that miRNAs play a role in the regulation of SOCS proteins.

1.3.7 miRNAs

The first non-coding RNA shown to have regulating activity was the *lin-4* gene in *Drosophila* in 1993 by Victor Ambros and colleagues [123]. miRNAs are a family of small, non-coding RNAs that have recently emerged as key post-transcriptional regulators of gene expression, in a sequence specific manner [124]. These *lin-4* RNAs were found to have antisense complementarity to multiple sites in the 3' untranslated region (3'UTR) of the *lin-14* gene and to regulate the amount of LIN-14 protein without affecting the level of *lin-14* messenger RNA (mRNA) [125]. Hundreds of miRNAs have since been discovered in the human genome and it is estimated that up to 30% of protein-coding genes are regulated by them [126]. miRNAs are generally 21-25 nucleotides in length and bind to target 3'UTRs through imperfect complementarity, negatively regulating target expression at the translational level; however, if the miRNA has perfect complementarity to the mRNA, it will specify direct cleavage [127].

1.3.8 Origin of miRNAs

MiRNAs are often contained within introns of protein coding genes or can be transcribed from their own promoters. Two processing events lead to the mature miRNA formation; primary miRNA (pri-miRNA) transcripts are processed into ~70-nucleotide precursors (pre-miRNA) and then the precursor is cleaved to generate the

21-25 nucleotide mature miRNA [128]. The cleavage of the pri-miRNA transcript, to release the pre-miRNA, is carried out by the enzyme Drosha inside the nucleus [129]. Exportin 5 (Exp5), a Ran-GTP dependent nucleo/cytoplasmic cargo transporter, is then required to transport the pre-miRNA from the nucleus to the cytoplasm [130]. Dicer, a member of the RNase III superfamily, cleaves the pre-miRNA ~19bp from the Drosha cut site resulting in a double stranded RNA, only one of which is the mature miRNA strand [131]. The double stranded RNA then dissociates and the mature miRNA strand is incorporated into the RNA induced silencing complex (RISC) complex, where the miRNA targets mRNAs by direct base pairing [132]. The 5' end of a miRNA, known as the 'seed' region (6-8 nucleotides), is the most important as it binds to a specific section in the 3'UTR of a target gene and mediates the function of mRNA degradation or translational repression, based on complementarity (Figure 1.7) [133]. Since miRNAs do not require perfect complementarity for target recognition, a single miRNA is able to target mRNAs of hundreds of distinct genes [134]. Equally as important, the impact on each regulated mRNA is subtle, but the combined effect is significant and produces measurable phenotypic results.

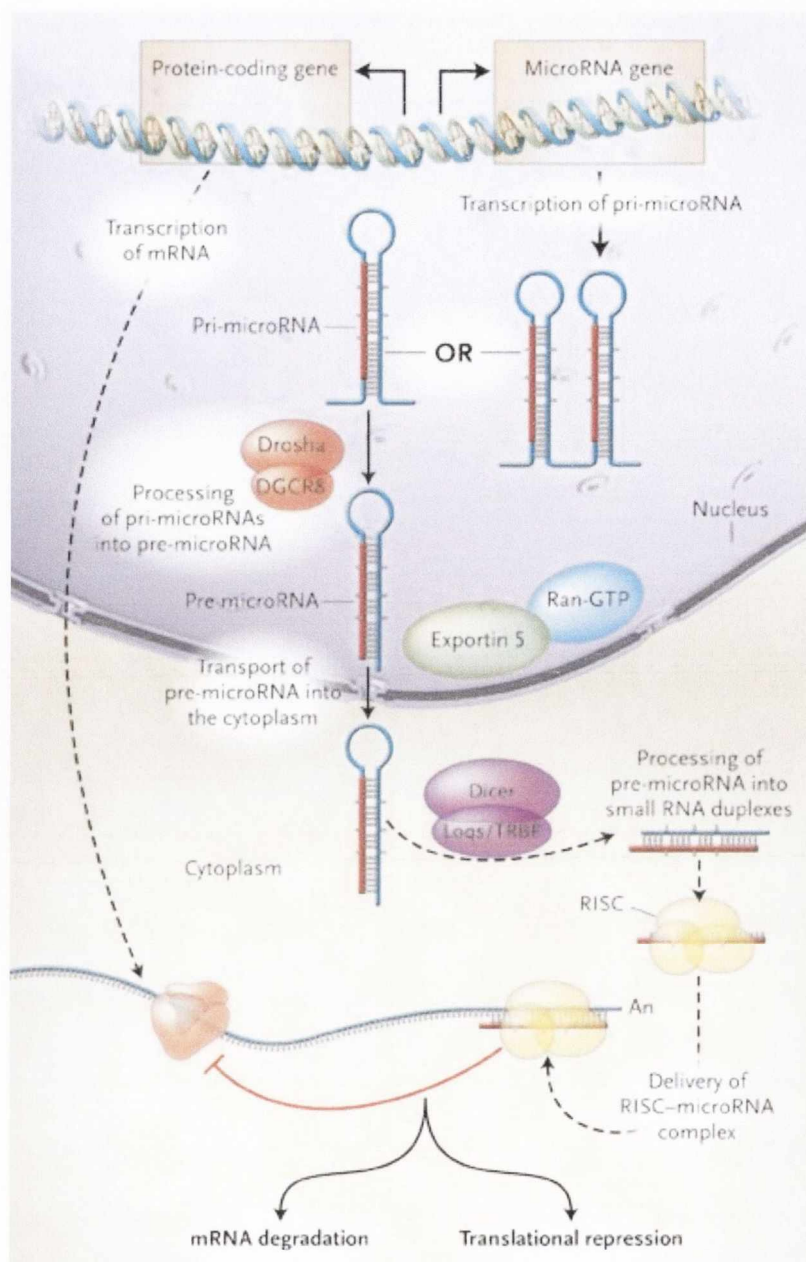


Figure 1.7: Origin and post-transcriptional suppression of miRNAs

Pri-miRNA transcripts are first processed into ~70-nucleotide stem-loop pre-miRNAs by the RNase III endonuclease Drosha, inside the nucleus. Exp5 then transports the pre-miRNAs to the cytoplasm where they are processed into miRNA:miRNA duplexes by the RNase III enzyme Dicer. The functional strand of the miRNA:miRNA duplex is preferentially assembled into the RISC complex, which subsequently acts on its target by translational repression or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target. Image taken from [135].

1.3.9 miRNA mediated regulation

Many miRNAs are evolutionarily conserved across multiple species indicating the importance of these molecules as regulators of critical biological processes; which include the immune response, cell-cycle control, metabolism, stem cell differentiation and human development [126]. Indeed, it is now apparent that abnormal miRNA expression or function is a common feature in many disease states, including cancer, heart failure and chronic inflammatory diseases, such as psoriasis [136]. Our knowledge of specific miRNAs and the roles they play in normal development as well as in disease states has increased dramatically since their discovery. For example, miR-146a/b was one of the first miRNAs shown to be involved in innate immunity. miR-146a is highly expressed in organs containing significant amounts of leukocytes, such as they thymus and the spleen [136]. It acts by regulating TLR signalling and cytokine responses [137]. Promoter analysis revealed miR-146a to be NF- κ B dependent and responsive to LPS, TNF- α and IL-1 β in THP-1 cells. Through luciferase reporter experiments, two key adaptor molecules in the TLR/IL-1 β pathway, TRAF6 and IRAK1, were identified as targets of miR-146a, suggesting it may function in a negative feedback loop to regulate TLR signalling, in response to bacterial products [138]. These results indicate that miR-146a expression may be important for preventing excess inflammation. Recent reports have shown that increased miR-146a expression is present in psoriatic lesional skin and at a higher level in psoriasis than atopic eczema skin samples, suggesting a role for miR-146a in the pathogenesis of psoriasis [136].

miR-155 is another well characterised miRNA whose function, investigated in transgenic mouse models, has been linked to various immune roles such as germinal centre response, B and T cell reactions and antigen presentation [137]. miR-155 is

reported to be induced by polyinosinic:polycytidylic acid poly(I:C) and the inflammatory cytokines IFN- β and IFN- γ , through TNF- α signalling, suggesting it to be a component of the innate immune response to viral and bacterial inflammatory mediators [139]. Several targets of miR-155 (FADD, RIPK1 and IKK ϵ) are involved in LPS signalling and miR-155 was found to repress the activation of the LPS/TNF- α pathway by enhancing TNF- α translation. This indicates that miR-155 could be involved in the negative regulation of the immune response to LPS [126]. Moreover, an additional essential role for miR-155 emerged in B and T cell responses when miR-155-deficient mice displayed defects in DC-T-cell interactions. DCs did not effectively present antigen to successfully activate T lymphocytes, furthermore, T cells exhibited an increased tendency to differentiate into Th2 type cells when activated [140]. Collectively these results indicate that miR-155 has established roles in innate as well as adaptive immunity.

The miRNAs, miR-17-92, appear in a genomic cluster as a single polycistronic transcript and encode six precursor miRNAs (miR-17, miR-18a, miR-19a, miR-19b1, miR-20a and miR-92-1) [141]. This miRNA cluster is of particular interest, as the sequences of the mature miRNAs and their organisation are highly conserved among vertebrates, suggesting that each miRNA in the cluster is of relevance. miR-17-92 was first linked to cancer, as it was found to be enhanced in lung cancer, chronic myeloid CD34⁺ cells and myc-rearranged lymphoma, and was subsequently verified to be an oncogene [142,143]. Additional functions, such as the regulation of haematopoiesis and immune regulation, have recently been assigned to the miR-17-92 cluster. Transgenic overexpression of the cluster resulted in an increase in B cell proliferation, plus survival of B and T cells, resulting in lymphoproliferative disease and autoimmunity in mice. The pro-apoptotic proteins Bcl-2-like protein 11 (BIM) and phosphatase tensin

homolog (PTEN) were identified as the functional targets of miR-17-92, mediating the phenotype [144]. Ventura *et al.*, found that miR-17-92 knockout mice exhibited severe developmental defects of the lung and heart and died within minutes of birth. The mice constitutively lacking the cluster displayed a severe block of B cell development linked to an increase in BIM levels, and thus apoptosis [145].

Since the discovery of these novel regulators, significant advances in relation to their function in normal biological processes and disease states have been made. A role for miRNAs in the regulation of TLR signalling has been identified. Furthermore, the miR-17-92 cluster is reported to be involved in cell proliferation and tumour growth, and could therefore be reasonably hypothesised to have a role in regulating the response to type I IFN. However, little is known about the individual miRNAs that compose the cluster. Could each of the miRNAs have additional, separate roles? Can they function alone? Further investigation into the regulatory roles of specific miRNAs will provide insight into the control of the immune system and increase our understanding of how immune homeostasis is maintained.

1.3.10 miRNAs and SOCS

SOCS1 and SOCS3 have been reported to interact with miR-19a and miR-203, respectively [136,146]. The 3'UTR of SOCS1 contains a complementary sequence to miR-19a and has been implicated in multiple myeloma (MM) pathogenesis. Pichiorri *et al.*, demonstrated that the up-regulation of miR-19a in MM could contribute to SOCS1 down-regulation and IL-6 activation at later stages in MM pathogenesis [146]. Moreover, virally induced miR-155 has been shown to target SOCS1 in macrophages, positively regulating the host antiviral response by promoting type I IFN signalling, thus suppressing viral replication [147]. However, in breast cancer cells, miR-155 plays

an oncogenic role by suppressing SOCS1, which results in the constitutive activation of STAT3 through the JAK-STAT pathway [148]. It is clear from these results that miRNAs can have not only a positive, but also a negative impact on health and disease. miR-19a is reported to be induced by IFN- α , suggesting it may play a role in the regulation of the JAK-STAT pathway and potentially enhance, or contribute to, the role of SOCS proteins [149].

SOCS3 is a conserved target of the skin and keratinocyte specific miRNA, miR-203. The up-regulation of miR-203 in psoriatic plaques was found to correlate with down-regulation of SOCS3 [136]. IL-6, also present in psoriasis lesions, induced constitutive STAT3 activation suggesting that the suppression of SOCS3 in psoriatic keratinocytes leads to the sustained activation of STAT3 signalling [136]. Contrastingly, results by Lena *et al.*, found that SOCS3 protein expression showed a significant, consistent increase during *in vitro*-induced differentiation in primary mouse keratinocytes, in parallel with miR-203, proposing that SOCS3 is not repressed by miR-203, despite good bioinformatic alignments [150]. These conflicting results indicate that although miRNAs have many predicted target genes, not all targets are functional and further verification is necessary to attribute a particular function to a specific miRNA.

Known miRNAs comprise 1-5% of all genes, making them an abundant class of negative regulators. Their high degree of conservation across species also indicates their importance in essential biological processes. It is probable that miRNAs work together with other mechanisms as an additional level of control or possibly to fine tune existing regulatory molecules. SOCS proteins are not only targets of miRNAs; SOCS1 and SOCS3 have been reported to be targeted by HCV, and are implicated in the resistance of viral clearance, which will be discussed next.

1.4 Inflammation and viral infection

1.4.1 HCV

HCV is a major public health concern globally as it is estimated to infect 180 million people. There is no vaccine against HCV and therapies are expensive and frequently ineffective. HCV was first discovered in 1989 and is a major cause of liver cirrhosis, chronic hepatitis and hepatocellular carcinoma, with an estimated 3-4 million new persons infected each year [151]. Since the introduction of anti-HCV screening of blood and blood products, post-transfusion transmission has become virtually non-existent and the primary mode of infection in developed countries is through shared needles by intravenous drug abusers [152]. Antiviral treatment for HCV consists of IFN- α and the nucleoside analogue Ribavirin, however effectiveness can be as low as 50% due to toxicity, viral resistance and genotype.

1.4.2 Genetic organisation of the HCV genome

HCV contains a ssRNA genome of ~9600 nucleotides in length. The viral genome consists of a 5' noncoding region (5'NCR) which includes an internal ribosome entry site (IRES) essential for cap-independent translation of the viral RNA, an open reading frame (ORF) that encodes the structural and non-structural (NS) proteins and a 3'NCR which is presumed to function in the initiation of minus-strand RNA synthesis (Figure 1.8) [153]. The structural proteins, which include the glycoproteins E1 and E2 and the core protein are the primary components of the virion. The core protein is found on the membranes of the ER or on the surface of lipid droplets and forms the viral nucleocapsid. It has been speculated that the interaction of the core protein with lipid droplets may affect lipid metabolism leading to lipid steatosis identified in many HCV

patients [151]. The envelope proteins E1 and E2 represent the building blocks of the viral envelope [152]. P7 forms oligomers and has cation channel activity which could have an important role in viral particle maturation and release.

The NS2-3 autoprotease cleaves the polyprotein between NS2 and NS3 and has been reported to be responsible for the replication of full-length HCV genomes *in vivo*. The NS3-4A complex is essential for downstream cleavage events in the NS region and contains an NTPase and helicase domain in the C-terminal of NS3. NS5A has a potential role in modulating IFN response. Mutations within the IFN sensitivity determining region (ISDR) of NS5A have been linked to an improved IFN- α therapy response. NS5B contains the key enzyme NS5B RdRp which is responsible for the synthesis of the complementary minus RNA strand and the subsequent synthesis of the genomic plus RNA strand. For this reason it has become a major target for antiviral intervention [152].

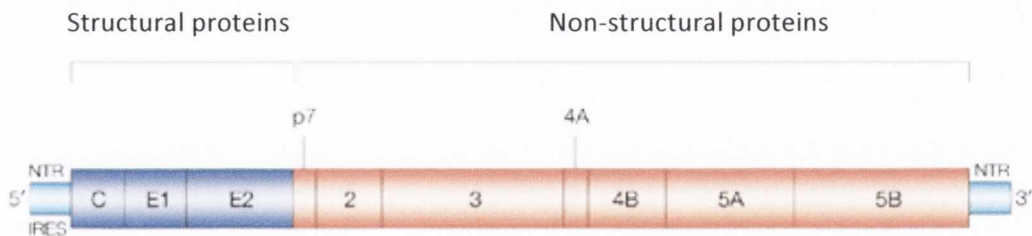


Figure 1.8: Schematic structure of the HCV genome

The 5'NCR consists of four highly structured domains and contains the IRES, while the 3'NCR consists of stable stem-loop structures. The central 9.6-kb ORF codes for a polyprotein of ~3200 amino acids. The HCV polyprotein is post-translationally cleaved by cellular and viral proteases into ten different products, with the structural proteins in blue [core (C), envelope glycoproteins (E1 and E2)] located in the N-terminal third and the NS [p7 and NS2–5] replicative proteins in the remainder. Image taken from [154].

1.4.3 The virus and its life cycle

HCV is a member of the *Flaviviridae* family of viruses and isolates can be classified into 6 genotypes, of which genotypes 1, 2 and 3 are most common. Within the HCV genotypes, many subtypes can be defined that differ in their nucleotide sequence by 20-25%, compared with >30% difference between genotypes [155]. This is most likely due to the high replicative activity combined with the poor proof-reading ability of HCV, as with all RNA viruses. The envelope glycoprotein, E2, undergoes rapid evolution to prevent recognition by the immune system, whereas the 5'NTR region is among the most highly conserved regions of the viral genome, and is used to distinguish between genotypes [155,156].

In the past, lack of an efficient cell culture system or small animal model permissive for HCV has limited the investigation of the replication cycle. However, advances such as the development of the replicon system, functional HCV pseudoparticles (engineered retroviral particles bearing functional HCV envelope proteins) and the production of recombinant infectious HCV in tissue culture has allowed for the study of HCV RNA replication in human Huh-7 hepatocyte cells *in vitro* and the observation of the interactions between viral components and the host cell [151].

HCV's entry into hepatocytes is mediated by clathrin dependent endocytosis and involves the tetraspanin cluster of differentiation 81 (CD81), the scavenger receptor class B type I (SR-B1) and the tight junction proteins claudin and occludin [153]. Viral fusion with the endosomal membrane introduces the HCV genome into the cytoplasm, where translation and replication are initiated. Translation is driven by the IRES in the 5'UTR and yields a single polyprotein of ~3200 amino acids consisting of 10 mature structural and NS proteins [157]. The structural proteins and p7 are processed by host

ER signal peptidases whereas the NS proteins are processed by the NS2-3 and NS3-4A proteases [157]. The expression of these proteins leads to the formation of replication complexes in the cytosol that catalyse the transcription of negative RNA strand, from which positive RNA strands are generated [158]. The viral RNAs and capsid proteins are then packaged into new viral particles and transported out of the cell to infect neighbouring liver cells (Figure 1.9).

Advances in the knowledge of HCV's life cycle have informed our understanding of the virus and how it manipulates the host defence system, to avoid detection and establish infection. It has also allowed us to study HCV's mechanisms of survival in more depth, and to develop novel therapies and preventative measures against it. An effective innate immune response is necessary for the elimination of HCV infection, however depending on viral genotype and the genetic background of the host, ~50% of people go on to develop chronic infection. Several mechanisms have been proposed to explain this; one in particular is HCV's ability to evade the host antiviral responses, which will be discussed next.

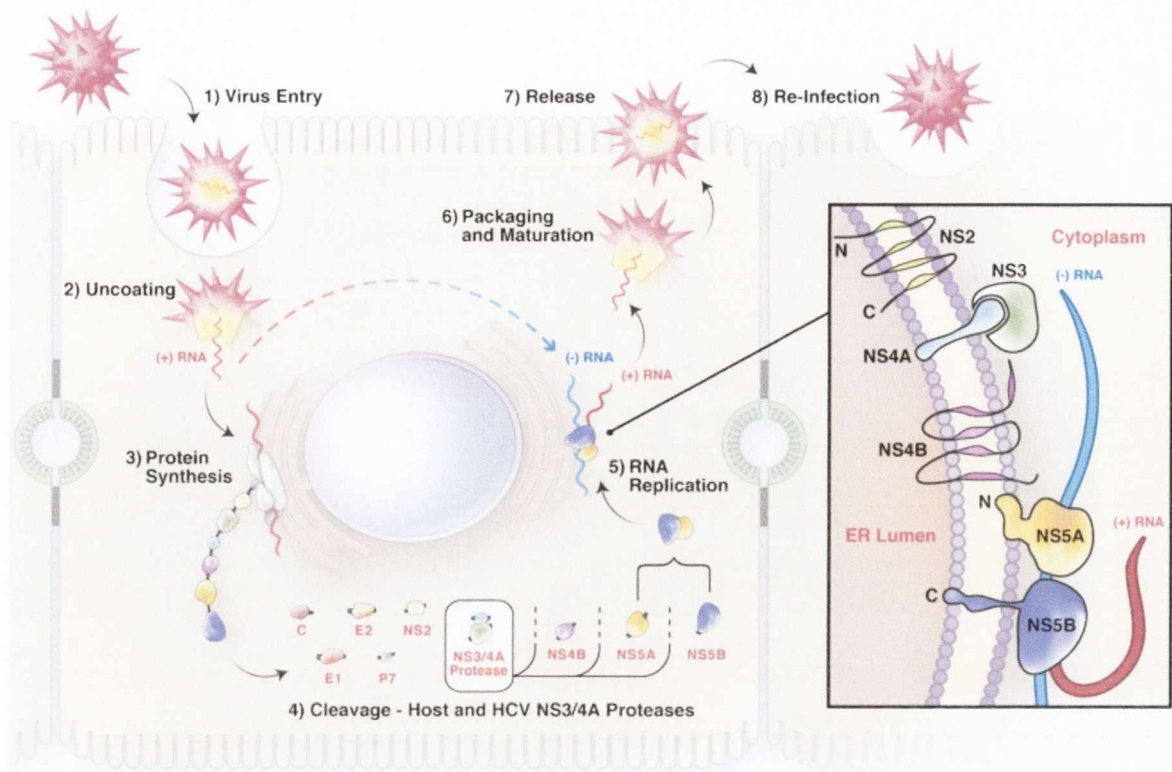


Figure 1.9: The life cycle of HCV

1. Virus Entry: HCV glycoproteins bind to a cell-surface receptor (e.g. CD81, SR-BI, low-density lipoprotein (LDL) receptor, claudin 1, and occludin) and internalise via clathrin-dependent endocytosis. **2. Uncoating:** Viral and cellular membranes are fused and a virus genome is released into the cytoplasm. **3. Protein Synthesis:** IRES mediated translation. **4. Cleavage – Host and HCV NS3/4A Proteases:** Processing HCV polyprotein by cellular and viral proteases. **5. RNA replication:** Once there is adequate RNA transcriptase, the viral RNA creates an antisense version of itself as a template for the creation of new viral RNA. **6. Packaging and Maturation:** Enveloped HCV virions carrying a newly synthesized viral genome form by budding into the ER lumen. **7. Release:** Release from host cell **8. Re-infection.** Image taken from [159].

1.4.4 HCV’s immune evasion strategies

HCV employs a multitude of strategies that interfere with innate immune responses, which subsequently impairs adaptive immunity, to facilitate persistent infection in the host. The majority of proteins that comprise the HCV genome have at least one mechanism of evading the immune response, outlined in Table 1.6.

HCV Proteins	Evasion Mechanism	REF
E2	Interacts with CD81 impairing NK cell activation	160
	Mutations in HVR allow for HCV variants and viral escape from neutralising E2 ab	160
	Acts as competitive substrate for eIF2α for PKR binding. Inhibits PKR activity	162
Core	Binds to complement receptor gC1qR, inhibits T-cell function	160
	Interacts with TNFR1, modulates the apoptosis of hepatocytes and lymphocytes	160
	Interacts with DEAD box protein 3	162
	Binds directly to STAT1 to prevent its phosphorylation and activation of ISGs	162
	Induces SOCS3, which negatively regulates Jak-STAT signalling pathway	162
	Activates NF-κB and AP-1 via TLR2	32
	Induces cytokines that are responsible for pDCs that produce low IFN	171
NS3	Binds to TBK1 to block association of TBK1 and IRF3	162
	Activates NF-κB and AP-1 via TLR2	32
NS3/4A	Cleaves IPS-1 and blocks signalling by RIG-1. Cleaves TRIF, blocks TLR3 signalling	162
NS4B	Blocks RIG-I and IPS-1 mediated signal transduction	162
NS5A	Inhibits PKR activity	162
	Induces IL-8, resulting in decreased ISG expression	162
	Binds to MyD-88, prevents cytokine induction in response to TLR ligands	162
	Blocks activation of 2',5'-OAS	
HCV gene products	Mutation of CD8+ T-cell epitope to avoid T-cell recognition	160
	Modulation of NK/T cell function	160
	Insufficient cytokine production	160

Table 1.6: Viral factors and their mechanisms of immune evasion

The high rate of HCV replication combined with a lack of RNA polymerase proof reading capability allows for the generation of highly related but genetically distinct viral variants, referred to as quasispecies [160]. Quasispecies variation results in a decrease in recognition by T lymphocytes and has been implicated in the resistance to

IFN- α therapy [161,162]. Mutations within E2's hypervariable region (HVR1) account for a significant amount of sequence diversity and allows for viral escape from the attention of neutralising antibodies [163]. Additionally, E2 disrupts the activity of the antiviral ISG, PKR, by competitively binding to eIF2 α and inhibiting the kinase ability of PKR [164].

Once inside the cell, HCV PAMPs trigger detection by PRRs predominantly TLR3 and RIG-I, which results in the transcription and activation of IFNs and cytokines necessary to activate host defence. However, HCV protease NS3-4A cleaves innate immune adaptor IFN- β promoter stimulator (IPS)-1 which causes it to delocalise from the mitochondrial membrane thereby abolishing RIG-I signalling, including IRF-3 activation [165]. The NS3-4A protease can also ablate TLR3 signalling by cleaving the TRIF adaptor protein [166,167]. The viral disruption of these PRRs has many implications downstream, including inhibition of the production of IFN- α , required to further amplify the magnitude and duration of the host response, suppression of CD4+ T cell activation by dysfunction of myeloid (m)DCs and deregulating the expression of numerous cytokines and chemokines whose production is dependent on NF- κ B [32,166].

PAMP induced release of IFNs and subsequent activation of the JAK-STAT signalling pathway is indispensable for host antiviral defence and resolution of viral infection. Not surprisingly, the attenuation of this antiviral pathway has been linked to HCV. HCV core protein has been reported to increase SOCS3 expression levels in cultured cells, which has been attributed to the enhanced activation of STAT1 by Bode *et al.*, and Lin *et al.*, and STAT3 by Yoshida *et al* [168-170]. This in turn leads to the negative regulation of the JAK-STAT pathway and inhibition of ISG production. Additionally, elevated hepatic SOCS3 levels have been linked to poor response to IFN- α therapy

[171]. Viral protein NS5A has been identified as an IFN antagonist and can inhibit PKR and induce CXCL8, resulting in reduced production of ISGs [164,172]. Preliminary data within our lab has shown that HCV degrades STAT3 thus preventing the up-regulation of essential ISGs required to combat infection.

HCV's subversion of innate immunity thwarts the adaptive immune system by failing to generate a robust immune response. Chronic HCV infection is associated with low frequencies of specific T cells and T cell responses however, this decline is poorly understood [173]. Frequent mutations in the viral genome can contribute to increased replication efficiency and reduced recognition by T cells, thus promoting viral escape [161]. Studies have indicated that DC function is altered during HCV infection [174], possibly due to an altered cytokine secretion profile, resulting in faulty T-cell activation, defective expansion of CD4⁺CD25⁺ regulatory T cells and a skewed differentiation of CD4⁺ T cells towards a Th2 phenotype, affecting CD4⁺ T-cell functionality [175,176]. HCV can also affect the development of an effective B-cell humoral response, further aiding immune evasion.

1.4.5 SOCS proteins and HCV

Because of the role of SOCS as powerful inhibitors of cytokine signalling through inhibition of the JAK-STAT pathway, they have become targets for viruses to exploit their function and escape the immune response [177]. Indeed HCV infection has been correlated with SOCS expression in a number of studies, including insulin resistance, inhibition of the JAK-STAT signalling pathway and resistance to IFN- α therapy, thus, it is not surprising that HCV research has focused on this area.

Evidence of insulin resistance and disturbances in glucose metabolism during HCV infection has been supported by clinical and molecular data. HCV core protein has been

shown to impair insulin signalling by up-regulating SOCS proteins, with subsequent ubiquitination and degradation of insulin receptor substrate (IRS)-1 [178]. Insulin resistance has also been linked to fibrosis progression and a reduced response to IFN- α therapy [179].

A study by Bode *et al.*, using HepG2 cells, found that over expression of HCV core protein leads to induction of SOCS3, which in turn inhibits IFN- α induced tyrosine phosphorylation, activation of STAT1 in hepatic cells and translocation of STAT1 to the nucleus [177]. Expression of SOCS3 is closely linked with STAT1 inhibition, indicating a functional association between the two. Furthermore Vlotides *et al.*, reported that the overexpression of SOCS1 and SOCS3 in HepG2 cells resulted in the inhibition of antiviral ISGs 2',5'-OAS and MxA [180]. Additionally, SOCS3 expression was found to be enhanced in association with a decrease in phosphorylated STAT1, in response to IFN treatment in chimpanzees infected with HCV [181]. These results indicate that HCV may be inducing SOCS1 and SOCS3 in order to suppress the IFN- α signalling pathway, and thus inhibit the production of essential antiviral ISGs necessary to clear HCV infection.

As predicted, elevated SOCS1 and SOCS3 levels have been associated with poor response to IFN- α therapy. Hepatic SOCS3 levels were found to be higher in non-responder patients than responders to treatment, which was also identified as the most powerful predictor of sustained virologic response [171]. Furthermore, increased levels of SOCS3 in liver tissue is associated with poorer treatment outcome in genotype 1b patients, suggesting that this particular genotype may exert its inhibitory effect on the IFN- α pathway via SOCS3 induction [182]. It may also explain why genotype 1 is most resistant to IFN- α therapy.

Although the majority of data is in concurrence with elevated levels of SOCS3 negatively affecting the IFN- α signalling pathway and response to therapy, there are some contrasting reports in the field. For example, while Huang *et al.*, noted an increase in SOCS3 levels in HCV infected chimpanzees treated with IFN- α , they reported the opposite pattern in a cohort of HCV infected individuals, with SOCS3 expression levels 11-fold higher in patients who responded to treatment compared with non-responders to treatment [181]. In addition, Shao *et al.* found a decrease in SOCS3 levels in HCV replicon-expressing cells and reported that overexpression of core protein had no effect on SOCS3. Interestingly, they found that SOCS3 overexpression in fact down-regulated core protein levels and decreased HCV replication, in an mammalian target of rapamycin (mTOR)-dependent manner [183].

As well as conflicting results over SOCS3, some studies have reported that SOCS1 expression is increased during HCV infection, while SOCS3 levels are unchanged. Imanaka *et al.*, reported that SOCS1 mRNA, but not SOCS3, was significantly higher in hepatic tissue of HCV infected patients and this correlated with a lower rate of sustained viral response (SVR) [184]. Miyoshi *et al.*, discovered that SOCS1 was suppressed in the liver of core gene transgenic mice and in HepG2 cells expressing core, while SOCS3 was conserved [185]. It is clear that further investigation into HCV's effect on SOCS proteins is required to define a distinguishing role for both SOCS1 and SOCS3 and the precise mechanism by which HCV induces them.

1.4.6 HCV induced pathology

Acute hepatitis infection refers to the first 6 months following exposure to HCV. Anti-HCV tests detect the presence of antibodies to the virus, indicating exposure to HCV. HCV RNA tests detect viral RNA and can distinguish between current and past infections. Of those who contract hepatitis C, ~10 to 25% successfully eradicate the virus with little or no noticeable symptoms [98]. The progression from acute to chronic infection, which occurs in ~75% of infected people, is in part mediated by pro-inflammatory cytokines, such as TNF- α and CXCL8. Patients also experience an increase in serum aminotransferase (ALT) levels consistent with the development of acute hepatocellular injury [186].

Indeed prolonged liver inflammation during chronic HCV infection can result in fibrosis, cirrhosis and eventually end-stage liver disease, however, analysis of the natural course of HCV infection by a Markov model indicated that the risk of developing cirrhosis over a 30-year period was 13–46% for men and only 1–29% for women, with a low lifetime prediction of end-stage liver disease complication and of liver-related deaths [187]. Furthermore, this slow progression to chronicity is not always associated with significant pathology, particularly in patients without co-factors such as obesity, excess alcohol or HIV co-infection, and many people with chronic hepatitis C have little clinical evidence of liver disease. If symptoms are present, they are usually mild, nonspecific and intermittent. In fact, population-based studies have revealed that ~50% of hepatitis C virus carriers have persistently normal ALT and two-thirds have mild histological liver lesions [187].

The mechanism for pathology associated with HCV infection, which is mild in many patients is not known, although inflammatory infiltrate and HCV's core protein have been implicated [188]. HCV infection results in the up-regulation of PRRs RIG-I and TLR3 which in turn mediate the secretion of pro-inflammatory cytokines. This process is tightly controlled by negative regulators and we propose that HCV may be hijacking this mechanism to regulate pro-inflammatory cytokine production, in order to limit pathology and prolong survival in the host.

Overall rationale underpinning thesis

Key Facts:

- SOCS1 and SOCS3 are established negative regulators of the JAK-STAT pathway and play a vital role in controlling the inflammatory response.
- miRNAs post-transcriptionally regulate the expression of $\geq 30\%$ of protein coding genes and have been implicated in the regulation of the immune response, therefore, miRNAs may regulate SOCS expression.
- SOCS proteins have been exploited by viruses to counteract the immune response and SOCS1 and SOCS3 are reported to be upregulated during HCV infection.
- SOCS1 and SOCS3 have been documented to regulate the TNF- α /NF- κ B signalling pathway.
- HCV infection can remain asymptomatic, often inflicting mild pathology in the host.
- HCV-induced SOCS could have a negative impact on the JAK-STAT and TNF- α /NF- κ B signalling pathway, thereby inhibiting the inflammatory response to infection.

Importance of investigation:

- Improved understanding of the regulation of SOCS1 and SOCS3 by miRNAs, cytokines and HCV will clarify its role in HCV infection and its effect on innate immune signalling pathways.

Hypothesis

We propose that miRNAs play a role in the regulation of the JAK-STAT pathway, via SOCS proteins. Additionally, it is our theory that HCV may induce SOCS3 to inhibit the JAK-STAT and TNF- α /NF- κ B signalling pathways and thus evade the immune response, limit pathology and establish persistent infection.

Overall Objective

To investigate the regulation of SOCS1 and SOCS3, by specifically focusing on the roles of miRNAs, cytokines and HCV, in order to reveal the gaps in knowledge of these powerful cytokine inhibitors.

Specific Aims:

1. miRNAs regulate up to 30% of protein coding genes. We aim to identify genes in the JAK-STAT pathway that may be regulated by miRNAs, in particular miR-19a, and any affects they may have on this signalling cascade.
2. Examine the expression, and the effect of IFN- α , on SOCS1 and SOCS3 in PBMCs from healthy and HCV infected individuals.
3. Examine the expression of SOCS1 and SOCS3 in Huh7 hepatocytes transfected with a HCV, to determine what affect the virus has on SOCS expression.
4. Determine whether TNF- α can induce SOCS1 and SOCS3, and if they can regulate TNF- α -signalling, in healthy and HCV+ PBMCs. Measure pro-inflammatory cytokine induction in response to TNF- α , to determine whether SOCS are involved in the regulation of the pro-inflammatory immune response during HCV infection.

Chapter 2

Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 General Materials

General laboratory chemicals were purchased from Sigma, USA, with the exception of Ficoll-Paque separation medium (Amersham, UK), RNA/DNA free H₂O, NuPAGE® MOPS SDS running buffer and Pre-cast BIO-RAD Criterion™ 4-12% Bis Tris gels (BioSciences, Dublin), agarose, BSA, EtBr (Fisher, USA), TRIzol®, DH5α competent E.Coli cells, One Shot® Top 10 chemically competent E.coli cells (Invitrogen, USA), Western blotting molecular weight marker (LI-COR, Germany), PVDF membrane, BCA protein assay reagent (Millipore, USA), Oligo dT, Omniscript RT Kit (Qiagen, Germany), SyBr Green, IFN-α2a (Roche, Switzerland), TaqMan® Fast Universal PCR Master Mix, TaqMan® MicroRNA Reverse Transcription Kit, MicroAmp Fast Reaction Tubes and Caps, MicroAmp Fast Optical 96-well Reaction plate (Applied Biosystems, USA), ELISA MAX™ Standard set human IL-6, IL-8 (CXCL8) kits (BioLegend, USA), CXCL10 ELISA kit and human recombinant TNF-α (Peprotech, UK), Pre-miR™ Negative Control #1 and hsa-miR-19a (Ambion, USA), recombinant human IL-6 (R&D Systems, USA).

2.1.2 Cell lines and plasmids

The human embryonic kidney (HEK293T) cell line stably transfected with the large T antigen and human monocytes (THP-1) were a kind gift from Prof. Luke O'Neill (Trinity College Dublin, Ireland). The hepatocyte cell line, Huh-7, expressing a T7 polymerase was a kind gift from Prof. Ralf Bartenschlager (University of Heidelberg, Germany). The HCV plasmid pBRTM/HCV1-3011 DNA construct was a kind gift

from Prof. Charles Rice (The Rockefeller University, USA). The T7 driven pBRTM/HCV1-3011, which is Tetracycline resistant, makes the HCV polyprotein (genotype 1b) and subsequent proteins but does not encode an RNA that is competent for HCV replicase driven RNA replication. Infectious HCV RNAs can now be produced in unlimited quantities. The empty vector pBR322 was purchased from Invitrogen, USA. The SOCS3 shRNA and scrambled control were purchased from Invivogen, USA. The SOCS3 positive control vector was a kind gift from Prof. Aki Yoshimura (Keio University, Japan).

2.1.3 Antibodies

For Western blotting, anti-SOCS3 was purchased from Abcam, UK, anti-pSTAT3 and anti-SOCS2 were purchased from Cell Signaling, USA, anti-SOCS1 was purchased from Invitrogen, USA, anti- β -actin and anti- γ -tubulin were purchased from Sigma, USA. Anti-I κ B α antibody was a kind gift from Prof. Ron Hay (Dundee University, UK).

2.1.4 Cell culture reagents

Dulbecco's Modified Eagle Media (DMEM), Roswell Park Memorial Institute (RPMI), Pen-Strep and trypsin-EDTA were purchased from BioSciences, Dublin. FCS was purchased from Analab, Ireland. Lithium heparin tubes for blood collection were purchased from BD Biosciences, USA. Trypan blue was purchased from Sigma, USA, Zeocin was purchased from Invivogen, USA and Lipofectamine 2000 was purchased from Invitrogen, USA.

RIPA Buffer:

- 10mls 1M Tris
- 15mls 5M NaCl
- 1ml 0.5M EDTA (pH 8)
- 5mls Triton X-100
- 5mls 10% SDS
- 464mls dH₂O

5X Blue Juice:

- 3mls 1M tris
- 5mls glycerol
- 1g SDS
- 1ml β -mercaptoethanol
- Bromophenol blue

10X Transfer Buffer:

- 30.0g tris
- 140.2g glycine
- Made to 1L with dH₂O

1X Transfer Buffer

- 100ml 10X transfer buffer
- 200ml methanol
- 700ml dH₂O

Trypan Blue

- 50ml PBS
- 0.4g trypan blue

2.2 Patient Samples

Patients attending the HCV out-patients clinic at St. Vincent's University Hospital, Dublin were recruited for this study. All patients were infected with HCV genotype 1 of

the virus. The majority of patients were female and contracted the virus after receiving contaminated anti-D-immunoglobulin from a single source between May 1977 and November 1978, others were infected resulting from a blood transfusion. All patients tested positive for antibodies to HCV using a third-generation enzyme immunoassay (Abbott Diagnostics) and a third-generation recombinant immunoblot assay (RIBA-3) (Chiron Corp.). None of the patients had received antiviral therapy at the time of entry to the study.

2.3 Ethics

Written informed consent was obtained from each patient and healthy volunteer and the study received ethical approval from the local Research and Ethics Committee at St. Vincent's University Hospital, in accordance with the guidelines of the 1975 Declaration of Helsinki.

2.4 PBMC isolation

PBMCs were isolated from 30ml of whole blood obtained from healthy and HCV infected donors using Ficoll-Paque™ PLUS and Leucosep® tubes. Ficoll-Paque™ PLUS is a sterile, ready to use density gradient medium for purifying lymphocytes in high yield and purity from small or large volumes of human peripheral blood. Leucosep® tubes contain a porous barrier incorporated into the centrifuge tube that prevents the mixture of the whole blood with the Ficoll-Paque™ PLUS. 15ml of Ficoll-Paque™ PLUS was added to the Leucosep® tube and centrifuged at 280g for 2mins. 15-20mls of blood was then added to the tubes and centrifuged at 1580g for 5mins with the brake off. During centrifugation, lymphocytes and PBMCs are separated from unwanted erythrocytes and granulocytes on the basis of their buoyant density, and

enriched in an interphase above the separation medium. When separation is complete, the barrier prevents recontamination of the enriched cell fraction during harvest. The PBMCs were then washed twice at 445g for 5mins with 20ml of RPMI-1640 medium. Cell pellet was resuspended in 1ml of RPMI, supplemented with 2% heat inactivated fetal calf serum (FCS), 250U/ml penicillin and 250µg/ml streptomycin, before counting using trypan blue.

2.5 Cell Culture

Huh7 cells (expressing a T7 polymerase) are a hepatocyte derived cellular carcinoma cell line and HEK293T cells are derived from human embryonic kidney cells. Both were grown in DMEM supplemented with 10% FCS, 2mM L-glutamine, 250U/ml penicillin and 250µg/ml streptomycin and cultured at 37°C in 5% CO₂ and 95% humidity. Huh7 cell medium was supplemented with 6µg/ml zeocin. Thp-1 cells are a derivative of a human acute monocytic leukemia cell line and are grown in RPMI supplemented with 10% FCS, 250U/ml penicillin and 250µg/ml streptomycin. Cells were passaged every 2-3 days when ~80-90% confluence was reached. Huh7's and HEK293T's are adherent cells, Thp-1 cells are in suspension. 10mls of trypsin was added to the Huh7/HEK293T cells for 10mins to detach cells from the flask. All cells were spun for 5mins at 285g and the pellet was resuspended in DMEM/RPMI before being returned to a clean, sterile T-175 tissue culture flask.

2.5.1 Stimulations

IFN-α was prepared from Roferon-A (IFN-α-2a, Roche) to a working concentration of 1000IU/ml using DMEM for cell lines and RPMI for PBMCs; 20ng/ml TNF-α (Peprotech) was diluted similarly. IL-6 was used at a working concentration of 10ng/ml

(R&D Systems). Cells were rested for 2h in media supplemented with 2% FCS, prior to stimulation, to synchronize cell cycle phase. The concentrations of cytokines and IFNs used were based on a combination of previous lab member's experimental design and publications [189-191].

2.5.2 Cell transfections

Cells were grown in 10% DMEM containing no antibiotics at a density of 0.2×10^6 in a 6-well plate, prior to transfection. Huh7 cells were transfected with 0.75 μ g of pBRTM/HCV1-3011 DNA construct (containing the structural and non-structural proteins of HCV genotype 1b) or pBR.322 empty vector (EV) for 16h using Lipofectamine 2000 (Invitrogen), according to the manufacturer instructions at vector: Lipofectamine ratio of 1:3.

HEK293T and Huh7 cells were transfected with 5.0, 7.5 and 10.0nM of pre-miR-19a, precursor molecule, and Mature MicroRNA Negative Control (MMNC) (Applied Biosystems) for 24h using Lipofectamine 2000 (Invitrogen), according to the manufacturer instructions. MMNCs are random sequence pre-miRNA molecules that have been extensively tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function. Precursor miR-19a is a synthetic microRNA molecule designed to mimic endogenous mature miR-19a.

Huh7 cells were transfected with 0.5 μ g of shRNA SOCS3 and scrambled control shRNA for 24h using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

2.6 RNA extraction

Total RNA was isolated from PBMCs, Huh7s and HEK293Ts following the Trizol reagent manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were resuspended in 1ml of Trizol and left at room temp for 5mins. 200µl chloroform was added and the samples were vortexed for 15 seconds and left at room temp for 3mins. Samples were centrifuged at 12,000g for 15mins at 4°C. Following centrifugation three phases were visible within the tube. The aqueous phase (containing RNA) was transferred to a fresh tube, being careful not to contaminate the solution with the other phases. 500µl isopropanol was added to the new tube, mixed and incubated at room temp for 10mins. Samples were centrifuge at 12,000g for 10mins at 4°C. Following centrifugation, supernatant was removed and pellet was washed with 1ml of 75% EtOH and vortexed. Samples were centrifuged at 7,500g for 5mins at 4°C. Supernatant was removed allowing remaining EtOH to air dry for 8mins. Pellets were resuspended in 30µl of RNase free water and transferred to a 60°C heating block and let sit for 5mins to dissolve the pellet. Samples were immediately transferred to ice to prevent RNA degradation. RNA yield and quality was then assessed using NanoDrop[®] ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer (Agilent Technologies) respectively, to ensure high quality RNA was extracted (Figure 2.1).

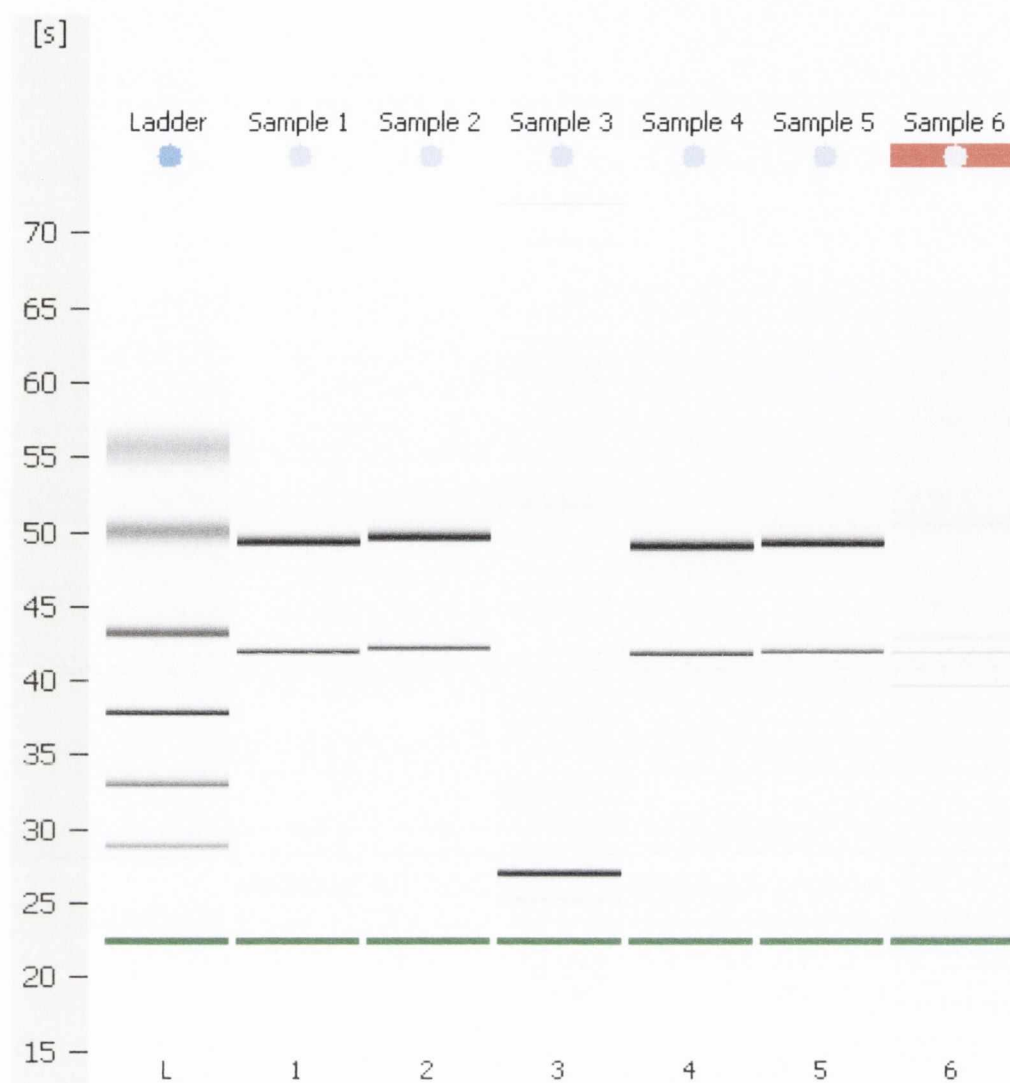


Figure 2.1: RNA quality of healthy PBMCs

A representative of Agilent Bioanalyser profile of 100ng and 5ng of RNA isolated from healthy PBMCs. Samples 1, 2, 4 and 5 show good quality RNA (100ng), detected by the 28S (top) and 18S (bottom) bands. Samples 3 and 6 indicate that the RNA (5ng) is degraded, by the range of visibly faint and incorrect sized bands.

2.7 quantitative Real Time PCR (qRT-PCR)

1µg of total RNA from each sample was reverse transcribed into cDNA using an OmniScript™ III first strand synthesis kit with oligo-dT primers according to the manufacturer’s instructions (Invitrogen Ltd, Paisley, UK). A total of 40ng cDNA, quantified using a NanoDrop® ND-1000 spectrophotometer, was subsequently used for each qRT-PCR reaction. Primers for real time qRT-PCR were designed, intron-spanning where possible, to ensure only cDNA made from mRNA extracted is being amplified and not contaminated genomic DNA. Primers were designed using Primer3 software [192] and commercially synthesised (Invitrogen Ltd, Paisley, UK). PCR amplification was performed using primer pairs specific for:

Gene	Upstream primer	Downstream primer
SOCS1	5'-TACTTGCTGGAACCATGTG-3'	5'-AGCTGCTACAACAACCAGGG-3'
SOCS2	5'-TCGGTCAGACAGGATGGTACTG-3'	5'-TTGGTCCAGCTGATGTTTAAACA-3'
SOCS3	5'-ATCCTGGTGACATGCTCCTC-3'	5'-CAAATGTTGCTTCCCCCTTA-3'
IL-6	5'-TCCACAAGCGCCTTCGGTCC-3'	5'-GTGGCTGTCTGTGTGGGGCG-3'
CXCL8	5'-GCAGAGGGTTGTGGAGAAGTTT-3'	5'-ACCCTACAACAGACCCACACAAT-
TNF-α	5'-TGGCCCAGGCAGTCAGATCA-3'	5'-GTAGGAGACGGCGATGCGGC-3'
CXCL10	5'-CCAATTTTGTCCACGTGTTGAG-3'	5'-GCTCCCCTCTGGTTTTAAGGA-3'
SOCS5	5' –TTCGTGCATGTTTTTTGAACCA-3'	5' –GGAGCCCATCAATTCCATCA-3'
Cul5	5' –TCTGTTTTCGGATGTGCATGCAGT-3'	5' –GCTCAGTACTCGTGCCTGTGCT-3'
RPS9	5'-TGACCGGGTGGTTTGCTTA-3'	5'-ATACTCGCCGATCAGCTTCAG-3'
RPS15	5'-CGGACCAAAGCGATCTCTTC-3'	5'-CGCACTGTACAGCTGCATCA-3'
GAPDH	5'-CCACCCATGGCAAATTCC-3'	5'-CAGCATCGCCCCACTTG-3'
β-Actin	5'-AGATGACCCAGATCATGTTTGAGA-	5'-CGTCACCGGAGTCCATCAC-3'
GUSB	5'-ATCCGCACTGTGGCTGTCA-3'	5'-CCAGCGAAGCAGGTTGAAGT-3'
PPIA	5'-GCAAATGCTGGACCCAAACAC-3'	5'-GCCATTCTGGACCCAAAG-3'
B2M	5'-GTGCTCGCGCTACTCTCTTT-3'	5'-GTCAACTCAATGTCGGATGGA-3'

Table 2.1: Forward and reverse primer sequences

Each reaction was carried out in duplicate, in a total volume of 25µl with 2µl of cDNA (20ng/µl), 12.5µl PCR master mix (Stratagene Corp, La Jolla, CA) and 10.5µl primer/H₂O. Optimal primer concentrations were determined by titrating 100, 300 and 900nM final concentrations and dissociation curves were examined for the presence of a single product. Real time qRT-PCR was performed the Sybr Green based fluorescent method and the MX3000P® quantitative PCR system (Stratagene Corp, La Jolla, CA) using the following cycling parameters: 95°C for 30s, 60°C for 1min and 72°C for 30s for 40 cycles, followed by amplicon dissociation

2.7.1 GeNorm data analysis

All gene amplifications for samples were normalised to Ribosomal protein 15 (RPS15), which was selected as the most stably expressed gene across samples from a panel of potential normalisers (β-Actin, RPS15, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A/Cyclophilin A (PPIA), β2 microglobulin (B2M), beta glucuronidase (GUSB) and RPS9 using GeNorm version 3.4.

2.7.2 qRT-PCR data analysis

Data analysis was carried out using the $2^{-\Delta\Delta C_t}$ method [193]. This involves comparing the cycle threshold (C_t) values of the samples of interest with a control, such as the unstimulated sample. The C_t is defined as the number of cycles required for the fluorescent signal to cross the threshold. The averages of the C_t values were calculated for all genes. The average C_t value of the gene of interest was then subtracted from the housekeeping gene; this is known as the ΔC_t value. To work out the $\Delta\Delta C_t$ value, relative to the unstimulated sample, the ΔC_t value of the unstimulated sample was taken away from the ΔC_t of the stimulated sample. This $\Delta\Delta C_t$ value was then entered into the

equation $2^{-\Delta\Delta CT}$ to get the relative gene expression of stimulated to unstimulated control. Therefore, from this equation the unstimulated control will be relative to itself, and as $2^0=1$, the unstimulated control is normalised to 1. Any negative changes in fold expression will be expressed as a decimal fraction. With every PCR run, a negative control without sample was included.

Statistical analyses were performed using non-parametric Mann-Whitney U test and Student t-tests on PBMCs and cell lines respectively, as implemented in version 5.01 of GraphPad Prism. P values of <0.05 were considered statistically significant.

2.8 Sample preparation for western blot

Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer (0.05M Tris-HCL pH 7.4, 0.15M NaCl, 1×10^{-3} M EDTA pH8) supplemented with aprotinin (5 μ g/ml), leupeptin (5 μ g/ml), PMSF (1mM) and Na_3VO_4 (1mM). Cells were lysed in RIPA buffer and incubated on ice for 15mins before centrifugation at 13,400g, 4°C for 15mins. Supernatants were then removed to a fresh tube. A bicinchoninic acid (BCA) protein assay was then carried out by adding 4.9ml of BCA reagent A to 100 μ l of BCA reagent B. 200 μ l of this mix was then placed in a 96-well plate with 5 μ l of supernatant and incubated at 37°C for 30mins. Absorbance was measured at a wavelength of 562nm. Protein concentration was calculated from a known BCA standard curve. All samples were normalised using the values generated from a standard curve in the BCA protein assay relative to their protein concentrations using RIPA buffer. 5x blue juice was added to each sample before they were boiled for 5mins at 95°C.

2.8.1 Western Blotting

Total lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which separates proteins based on their molecular weight. Samples were loaded on to a pre-cast 4-12% Bis-Tris Criterion Gel with NuPAGE® MOPS SDS running buffer. A molecular weight ladder was run alongside the samples. Gels were run at 90V until the proteins had run through the stacking gel; the voltage was then increased to 200V until the proteins had reached the end of the gel. Protein was transferred from the gel to a polyvinylidene difluoride (PVDF) membrane using semi-dry transfer. The PVDF membrane was soaked in methanol for 20seconds to activate it, washed in dH₂O for 2mins before being left to soak in transfer buffer. Blotting pads were also left soaking in transfer buffer. The gel was left in transfer buffer for at least 5mins prior to being assembled on the transfer machine. The semi-dry transfer was set up as follows, blotting pad, PVDF membrane, gel and blotting pad. This ran for 90mins at 20V. The membrane was then blocked for 60mins with 5% powdered milk in phosphate buffered saline tween 20 (PBST) (0.15% Tween-20 in PBS). For the pSTAT3 protein, the membrane was blocked in 3% bovine serum albumin (BSA).

Primary antibodies were diluted 1:1000 in 5% milk or 3% BSA and left incubating at 4°C overnight. The following morning blots were washed 3 times in PBST for 10mins before being incubated at room temp with relative secondary antibody, made to a concentration of 1:5000 with milk or BSA, for 1hr. Proteins were analysed on an Odyssey Infrared Imaging System.

Primary Antibody	Dilution	Secondary Antibody
SOCS1	1:1000	Goat anti-mouse
SOCS2	1:1000	Goat anti-rabbit
SOCS3	1:1000	Goat anti-rabbit
pSTAT3	1:1000	Goat anti-rabbit
γ -Tubulin	1:1000	Goat anti-mouse

Table 2.2: Antibodies used for Western blotting

2.9 Plasmid Transformation

Exogenous genetic material can be introduced into competent bacterial cells through the process of transformation. Transformation of plasmid DNA containing an origin of replication into competent bacterial cells allows for the rapid production of large quantities of plasmid DNA.

To transform DH5 α cells, a competent *Escherichia coli* (E.Coli) bacterial strain, 2 μ l of plasmid DNA was added to 10 μ l of DH5 α cells. Aliquoted DH5 α cells were stored at -80°C and when required, thawed on ice. Plasmid DNA-DH5 α mix was swirled gently and incubated on ice for 2mins. DH5 α cells were then heat shocked by incubating at 42°C for 45secs; this promotes uptake of plasmid DNA. The mix was incubated on ice for a further 2mins. 100 μ l of pre-warmed Luria-Bertani (LB) was added and the LB mix was plated onto LB agar plates containing either 100 μ g/ml ampicillin or 12.5 μ g/ml tetracycline, depending on the vector being transformed.

To transform One Shot® Top10 chemically competent E.Coli cells, a 10 μ l aliquot of Top10 cells were thawed on ice and 2 μ l of DNA mix was added. This mixture was incubated on ice for 30mins before the bacterial cells were heat shocked at 42°C for

45secs. The reaction was incubated for a further 3mins on ice and 100µl pre-warmed LB broth. The reaction was shaken at 200rpms for 1h at 37°C, before being plated onto LG agar plates containing 100µg/ml ampicillin. LB agar plates were incubated for 12-16h at 37°C. Uptake of plasmid DNA was confirmed by the presence of bacterial colonies on agar plates. Positive and negative controls were used for each transformation

2.9.1 Plasmid preparation and purification

Plasmid Mini-prep:

A single colony was added to 3mls LB broth containing 100µg/ml ampicillin and grown for 16h at 37°C while being shaken at 200rpms. 500µl of each culture was added to 500µl of glycerol and stored for future use at -80°C. The remainder of the cultures were mini prepped to isolate the plasmid DNA.

For purification of small amounts of plasmid DNA, Promega PureYield™ plasmid miniprep system (Promega Corporation, Madison, WI, USA) was used. 2.5ml of bacterial culture was centrifuged at 16,000 x g for 1min. Supernatant was discarded and cell pellet was resuspended in 600µl of NF-H₂O. 100µl Cell Lysis Buffer was added and the reaction was mixed by inverting 6 times. Within 2mins, 350µl pre-chilled Neutralisation Solution was added and the reaction was mixed thoroughly by inverting the tube. The reaction was centrifuged at 16,000 x g for 3mins before the supernatant was transferred to PureYield™ Minicolumn, which was then centrifuged at 16000 x g for 1min. The flow-through was discarded and 200µl of Endotoxin Removal Wash was added to the column. The column was centrifuged at 16000 x g for 1min before addition of 400µl Column Wash Solution and being centrifuged at 16000 x g for 1min. The minicolumn was transferred to a fresh, sterile 1.5ml eppendorf and 30µl of Elution

buffer was carefully added to the column. After leaving this to stand at RT for 1min, the column was centrifuged at $16000 \times g$ for 1min to elute plasmid DNA. Plasmid concentration was assessed using a NanoDrop[®] ND-1000 spectrophotometer.

Plasmid maxi-prep:

A single colony of transformed bacteria or 100µl of glycerol plasmid stock was added to 3mls LB broth and shaken at 200rpm at 37°C for 8h. This starter culture was then added to 150ml LB and shook at for a further 12-16h at 200rpm at 37°C. 500µl of culture was added to 500µl of glycerol and stored at -80°C. A Promega PureYield[™] Plasmid Maxiprep System was used to isolate plasmid DNA. Cells were pelleted by centrifugation at $5000 \times g$ for 10mins at RT and supernatant was discarded. Pellets were resuspended in 12ml Cell Resuspension Solution. 12ml Cell Lysis Solution was added and mixed by gently inverting 3-5 times followed by incubation at RT for 3mins. 12ml Neutralisation Solution was added to the mixture, which was then inverted 10-15 times. Lysates was centrifuged at $14000 \times g$ for 20mins at RT and added to a PureYield[™] Clearing Column, which was placed on a PureYield[™] Maxi Binding Column. A vacuum was applied until all liquid had passed through both columns. The clearing column was then removed. To wash the binding column, 5ml Endotoxin Removal Wash was added and a vacuum was applied until the liquid has passed through the column. 30ml of Column Wash solution was added and a vacuum was applied until the solution had passed through, after which vacuum was applied for an additional 5mins to ensure the column membrane was dry. Plasmid DNA was retrieved from the column by eluting in 1ml of NF-H₂O, which was added to the column, incubated at RT for 1min before vacuum being applied. Eluted DNA was retrieved in a sterile 1.5ml eppendorf. Plasmid concentration was assessed using a NanoDrop[®] ND-1000 spectrophotometer.

2.10 RT-PCR to detect microRNAs

2.10.1 Generating cDNA using Reverse Transcription PCR (RT-PCR)

RNA samples were diluted to 100ng/μl and the Applied Biosystems TaqMan micro-RNA reverse transcription kit was used with specific stem-loop primers for miR-19a to generate cDNA according to the manufacturer's instructions. The 15μl reaction consisted of 1.5μl 10X reverse transcription buffer, 0.15μl 100mM dNTP mix, 0.19μl RNase inhibitor, 1.0μl MultiScribe reverse transcriptase, 3.0μl 5X miRNA specific stem-loop RT primer, 6.16μl RNase free dH₂O and 3μl of the 100ng/μl stock RNA. Reactions were placed in Fast Reaction Tubes (Applied Biosystems) and carried out in a thermocycler (Applied Biosystems Veriti) under the following conditions: 16°C for 30mins, 42°C for 30mins, 85°C for 5mins and 4° indefinitely.

2.10.2 miRNA Specific Real Time PCR amplification

Applied Biosystems TaqMan miRNA specific probes were used to quantify the levels of miR-19a in a sample of cDNA. The Applied Biosystems 7900 Fast system was used according to the manufacturer's protocol. Reactions were assembled as follows: 5μl 2X TaqMan Universal Mastermix, 2.1μl RNase free dH₂O, 0.5μl 10X miRNA specific primer/probe mix and 2μl miRNA-specific RT product. The 7900 Fast cycling program was as follows: 95°C for 20seconds and 60°C for 30seconds for 40 cycles. miRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method using RNU6B as an endogenous control. Samples were carried out in triplicate.

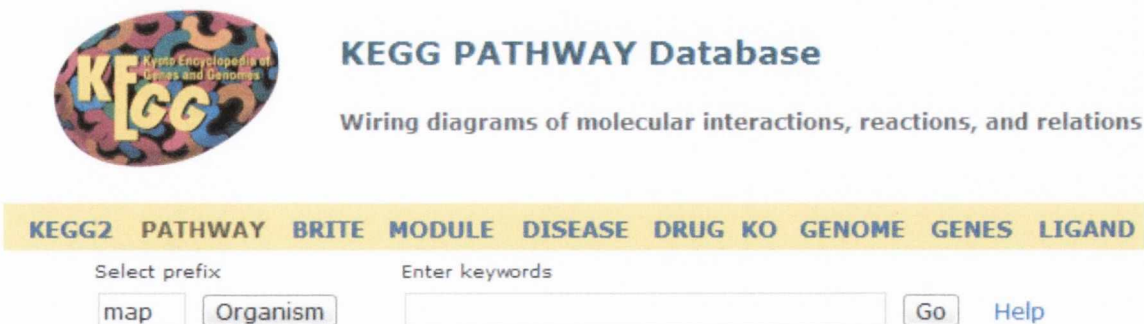
2.11 Enzyme-linked immunosorbent assay (ELISA)

(A) CXCL10 (Peprotech) and (B) CXCL8 (BioLegend) sandwich ELISA kits were used to measure cytokine responses to TNF- α . 96-well polystyrene microplates were coated with 100 μ l/well of specific capture antibody (diluted according to the protocol) in 1X PBS (A) and coating buffer (B) and incubated at room temp (A) and 4°C (B) overnight. The following day the plates were washed with 1% (v/v) PBS-Tween2000 by immersing plates into the wash buffer and completely removing the liquid by inverting each plate 3-4 times and then firmly tapping the plate upside down on absorbent paper. The plates were then blocked for 2h (A) and 1h (B) with 300 μ l of reagent diluent, (1% (w/v) bovine serum albumin (BSA) in PBS), to prevent non-specific binding. The plates were then washed as described above before 200 μ l of sample supernatant was added to the well and incubated at room temp for 2h. Samples were used neat for CXCL10 and diluted 1/20 in reagent diluent for CXCL8. Standards were diluted according to the manufacturer's protocol. Samples were then discarded and the plates were washed as before. 100 μ l/well of detection antibody (diluted according to the protocol) was then added to the plates for 1-2h. After washing, 100 μ l of diluted Avidin-HRP was added to each well for 30min. Plates were then washed 5 times to help minimise background. 100 μ l of TMB substrate solution was then added for ~15-30min and incubated in the dark. The reaction was stopped with the addition of 100 μ l of 1M H₂SO₄. Colour development was read on a VERSA Max microplate reader (Molecular Devices) at 450nm.

2.12 Bioinformatic Analysis

We utilised publicly available software and bioinformatic tools to search groups of genes for miR-19a targets. These included the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY database, InnateDB and TargetScan. KEGG was assembled by Kanehisa Laboratories in 1995 and remains a database resource for understanding high-level functions and utilities of the biological system, such as the cell, from genomic and molecular-level information [194]. In particular, KEGG PATHWAY is a collection of manually curated pathway maps representing knowledge on the molecular interaction and reaction networks and was used to identify proteins involved in JAK-STAT signalling (Figure 3.2). We compiled a list of these proteins by carrying out the following steps.

Step 1: Go to the KEGG PATHWAY website



Step 2: Scroll down to Signal Transduction and select Jak-STAT signalling pathway

3.2 Signal Transduction

Two-component system

MAPK signaling pathway

MAPK signaling pathway - fly

MAPK signaling pathway - yeast

ErbB signaling pathway

Wnt signaling pathway

Notch signaling pathway

Hedgehog signaling pathway

TGF-beta signaling pathway

VEGF signaling pathway

Jak-STAT signaling pathway

Calcium signaling pathway

Phosphatidylinositol signaling system

mTOR signaling pathway

Plant hormone signal transduction

Two-component system

Step 3: This gave us a list of options of which we selected Pathway entry.



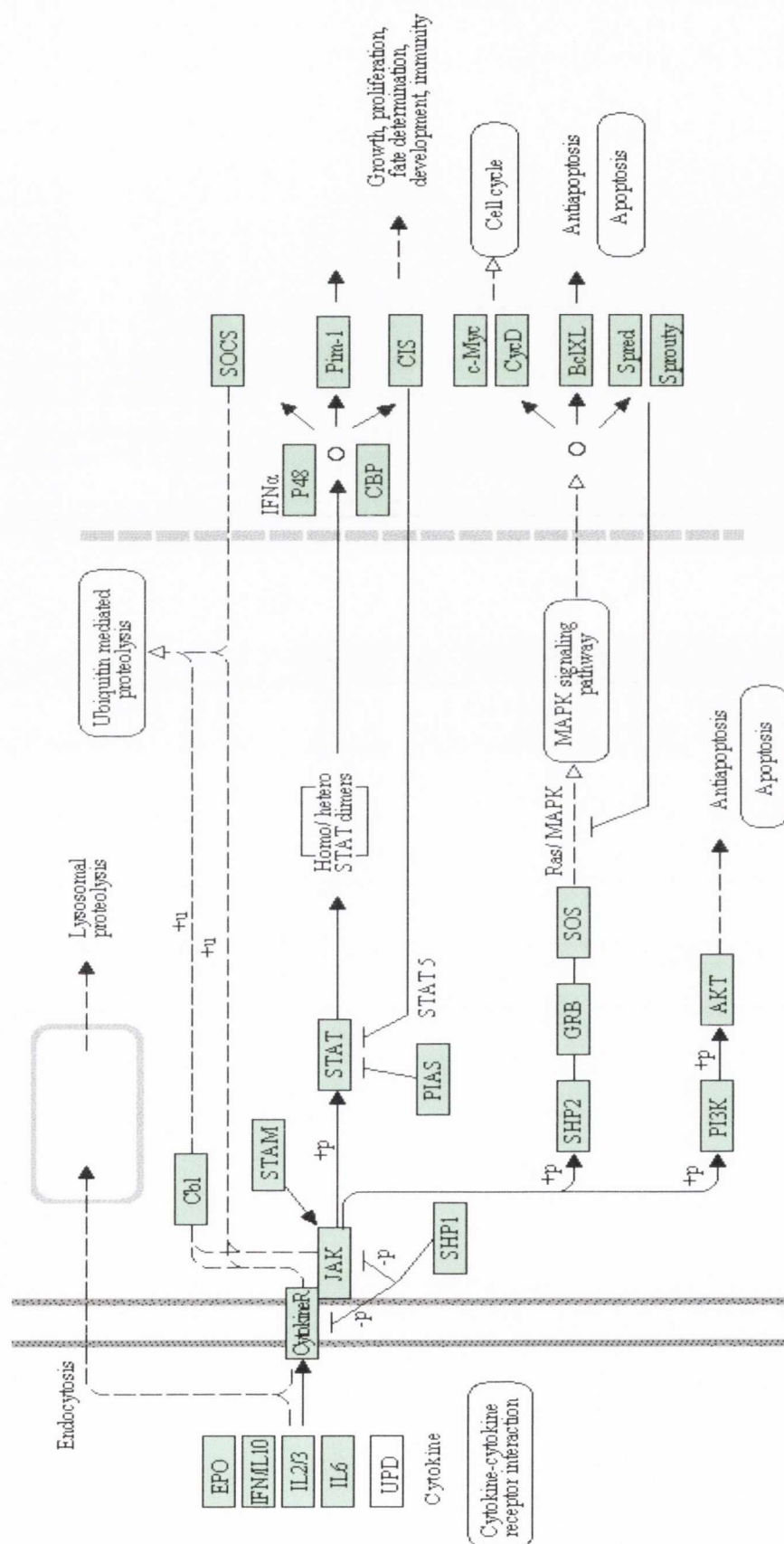
Jak-STAT signaling pathway - Homo sapiens (human)

[Pathway menu | Organism menu | Pathway entry | Download KGML | Hide description | User data mapping]

Step 4: We were then provided with a list of all proteins involved in this pathway, which we exported to excel.

Gene	7173	TPO; thyroid peroxidase (EC:1.11.1.8) [KO:K00431] [EC:1.11.1.8]
	2056	EPO; erythropoietin [KO:K05437]
	1442	CSH1; chorionic somatomammotropin hormone 1 (placental lactogen) [KO:K05438]
	2689	GH2; growth hormone 2 [KO:K05438]
	2688	GH1; growth hormone 1 [KO:K05438]
	5617	PRL; prolactin [KO:K05439]
	3458	IFNG; interferon, gamma [KO:K04687]
	3449	IFNA16; interferon, alpha 16 [KO:K05414]
	3447	IFNA13; interferon, alpha 13 [KO:K05414]
	3444	IFNA7; interferon, alpha 7 [KO:K05414]
	3443	IFNA6; interferon, alpha 6 [KO:K05414]
	3451	IFNA17; interferon, alpha 17 [KO:K05414]
	3442	IFNA5; interferon, alpha 5 [KO:K05414]

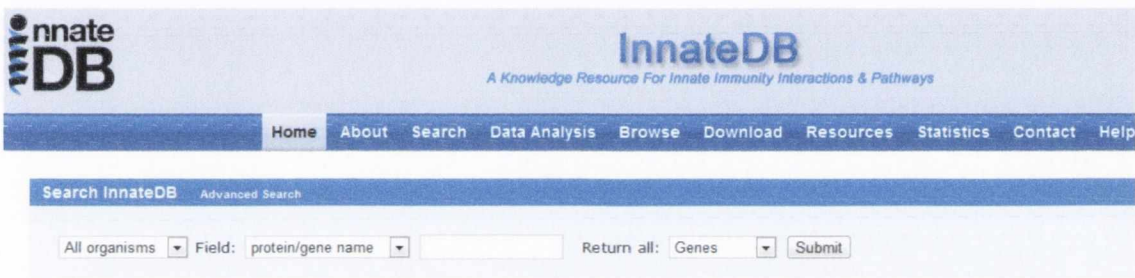
JAK-STAT SIGNALING PATHWAY



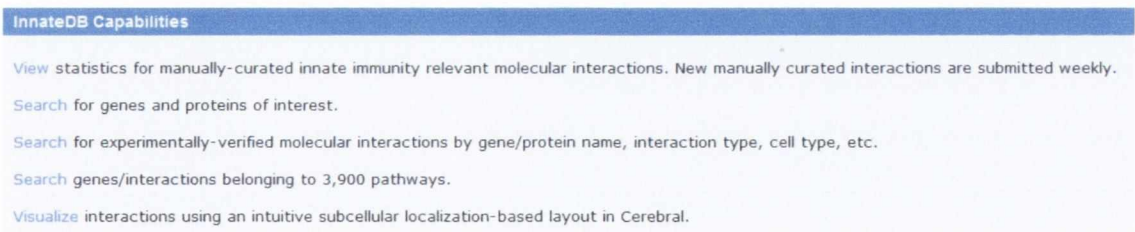
04630 3/31/09
(c) Kanehisa Laboratories

InnateDB is a publicly available database with a goal to provide a manually-curated knowledgebase of the genes, proteins and particularly, the interactions and signalling responses involved in the mammalian innate immune response [195]. InnateDB integrates interaction and pathway information from several major publicly available databases thereby capturing an improved coverage of innate immunity interactions. InnateDB was used to identify genes in the JAK-STAT pathway and their interactions by carrying out the following steps.

Step 1: Go to the InnateDB website.



Step 2: Scroll down to locate Search genes/interactions belonging to 3,900 pathways



Step 3: Here we selected the Jak-STAT Signaling Pathway (Human)

Pathway Advanced Search

Search interactions or genes by pathway [\(Help\)](#)

Select an example pathway from the list:

Jak-STAT Signaling Pathway (Hun

▼

OR search pathway name here:

OR enter external pathway database ID:

Select

▼

Return a list of:

Genes

▼

Only return interactions within the pathway:

☐ Yes

☒ No

Submit

Step 4: We were then provided with a list of proteins involved in the JAK-STAT pathway and their interactions. We exported this to excel and combined this with our results from the KEGG PATHWAY.

Viewing genes 1 to 20 of 154 hits matching query (INNATEDB accession: '568')

Page(s): 1 2 3 4 5 6 7 8 [Next]

InnateDB ID	Ensembl Gene ID	Organism	Chromosome	Gene symbol	Gene name	Experimentally validated interactions
IDBG-17489	ENSG00000113594	Homo sapiens	5	LIFR	leukemia inhibitory factor receptor alpha	11
IDBG-74884	ENSG00000147168	Homo sapiens	X	IL2RG	interleukin 2 receptor, gamma	28

TargetScan (version 5.0) is online software provided by MIT for the prediction of miRNA targets [196]. TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites in the 3'UTR of target genes that match the seed region of each miRNA. This software was used to identify the proteins that contain a target sequence for miR-19a in their 3'UTR by carrying out the following steps. We combined the list of JAK-STAT proteins with the list of miR-19a target proteins and identified the overlapping genes.

Step 1: Go to the TargetScan website; enter SOCS3 and other genes of interest



TargetScanHuman

Prediction of microRNA targets

Release 6.1: March 2012

Search for predicted microRNA targets in mammals

[\[Go to TargetScanMouse\]](#)
[\[Go to TargetScanWorm\]](#)
[\[Go to TargetScanFly\]](#)

1. Select a species

AND

2. Enter a human Entrez Gene symbol (e.g. "LIN28A")

Step 2: We were provided with the list of predicted miRNA's in the 3'UTR region of SOCS3



Step 3: We selected miR-19a and scrolled down to find out more about the binding site and its conservation.

Conserved		predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contribution	3' pairing contribution	local AU contribution	position contribution	TA contribution
Position 1561-1568 of SOCS3 3' UTR	5' ...CAAUCCAAGUCAAACTUUUGCACA... hsa-miR-19b	3' ...AGUCAAAACGUACCUAAACGUGU	8mer	-0.247	0.003	-0.048	-0.096	0.008
Position 1561-1568 of SOCS3 3' UTR	5' ...CAAUCCAAGUCAAACTUUUGCACA... hsa-miR-19a	3' ...AGUCAAAACGUAUCAAAACGUGU	8mer	-0.247	0.003	-0.048	-0.096	0.008

Chapter 3

Regulation of SOCS3 by miRNA

CHAPTER 3: REGULATION OF SOCS3 BY MICRORNA

3.1 Introduction

The JAK-STAT pathway, conserved in all vertebrates, is the principal signalling cascade to mediate cytokine-receptor derived signals. It is responsible for facilitating important biological mechanisms including inflammation, cell proliferation, maintenance of homeostasis, cell differentiation and antiviral activity, and is stimulated by receptor binding of many cytokines including the type I IFNs and interleukin families. Activation of JAKs leads to STAT phosphorylation, dimerisation and translocation to the nucleus where they bind responsive DNA elements, often inducing mediators such as pro-inflammatory cytokines and ISGs. Activation of this pathway is tightly regulated to control its onset and termination. SOCS proteins silence the pathway by 1) acting as pseudo-substrates that block JAK kinase activity, 2) binding to the receptor to prevent STAT interaction and 3) targeting proteins for proteasomal degradation [197]. Additional regulators of this pathway include SH2-containing phosphatase-1 (SHP-1) and SHP-2, and protein inhibitors of activated STATs (PIAS) [198]. The association of SHP proteins with the phospho-tyrosine residues of JAKs and STATs, triggers activation of the phosphatase domain and subsequent dephosphorylation of the substrate, rendering STATs inactive. The PIAS family of regulators have small ubiquitin-like modifier (SUMO)-E3-ligase activity that allows them to interact with STATs and negatively control their activity (Figure 3.1) [198].

Dysregulation of tyrosine kinases is associated with cancer and constitutive activation of JAK2 and STATs is thought to mediate neoplastic transformation and stimulate abnormal cell proliferation in various malignancies [198]. A mutation, V617F, in the gene that codes for JAK2 protein is found in the majority of patients with

polycythaemia vera and in approximately 50% of patients with essential thrombozythemia and idiopathic myelofibrosis [199]. Constitutively activated STATs can promote tumourigenesis by inducing deregulated cell growth and/or preventing apoptosis. Furthermore, abnormalities in the regulators of the JAK-STAT pathway have been detected in myeloproliferative disorders. For example, loss of PIAS function is related to anaplastic lymphoma kinase-positive T/null-cell lymphoma and myelodysplastic syndrome, while loss/mutation of SOCS1 expression results in primary mediastinal B-cell and Hodgkin's lymphoma [198].

Epigenetic silencing of the SOCS3 gene by methylation of CpG promoter islands has been linked to HCC and is likely to contribute to growth and survival of several other types of cancer [199].

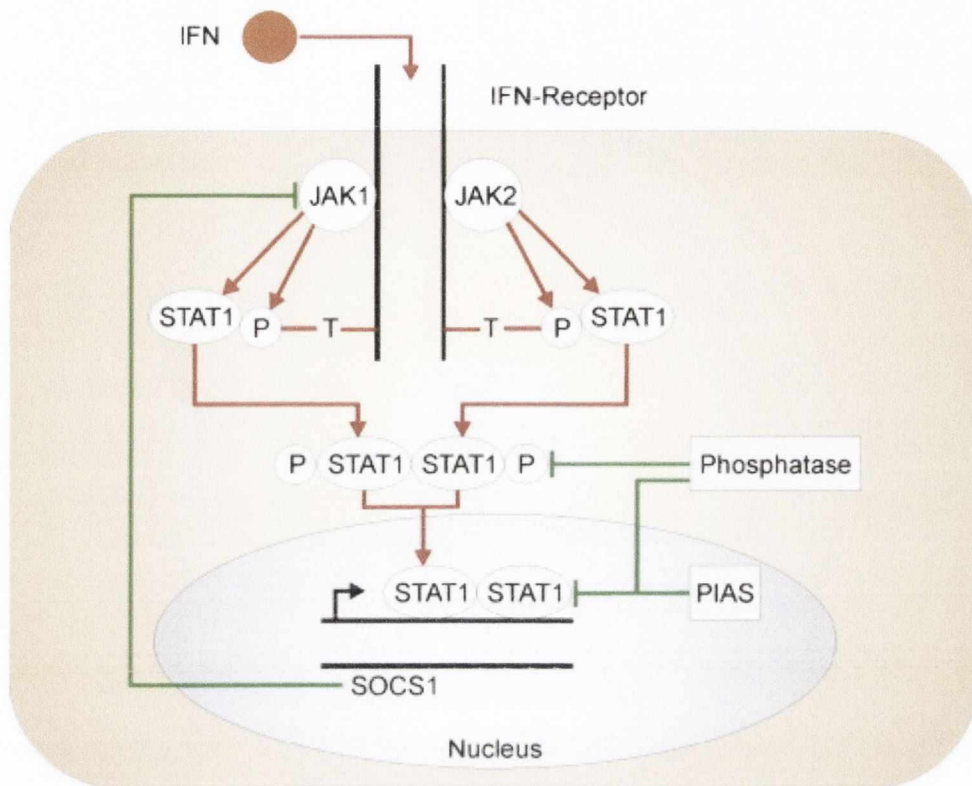


Figure 3.1: Negative regulators of the JAK-STAT pathway and their mechanisms of action

Cytokine stimulation of the JAK-STAT pathway leads to the activation of JAKs, which bind to and phosphorylate the receptor subunit, and allow the recruitment of STATs. STATs are phosphorylated and translocate to the nucleus where they initiate transcription. The JAK-STAT pathway is under strict regulation by SOCS proteins, tyrosine phosphatases and PIAS. Phosphatases dephosphorylate JAKs, cytokine receptors and STATs while SOCS proteins can inhibit the kinase activity of JAKs, prevent STATs from binding to the receptor and target them for proteasomal degradation. Image taken from [199].

miRNAs are small non-coding RNAs that regulate gene expression through mRNA degradation or translational repression, depending on base complementarity in target mRNAs. They have been identified in a multitude of species including viruses, plants, mice and humans, and it is increasingly clear that they are involved in regulating gene expression during essential biological processes such as cell cycle, differentiation, growth and apoptosis. miRNAs are highly conserved and loss of function studies have revealed that mice lacking Dicer, an enzyme essential for the production of mature miRNAs, die embryonically and lack multipotent stem cells [126]. A role for miRNAs in the regulation of immune responses has recently been documented where the presence of miR-181a in the thymus and miR-223 in the bone marrow were implicated in the differentiation of pluripotent hematopoietic stem cells [200]. Additionally, miR-155 and miR-146a have been shown to be induced by viral and bacterial pathogens respectively, as well as inflammatory cytokines, suggesting them to be components of the innate immune response [126]. Furthermore, miRNAs have been implicated in antiviral defence. IFN- β induces the expression of miR-196 and other miRNAs which contain sequence matches in the HCV genome, which has been shown to attenuate viral replication in infected liver cells [133].

Evidence for the involvement of miRNAs in inflammation was demonstrated by Sonkoly *et al.*, who identified a set of miRNAs associated with the chronic inflammatory diseases, psoriasis and eczema [136]. miR-203 was identified as a skin specific miRNA expressed in keratinocytes and up-regulated in psoriatic plaques. Using bioinformatic tools and prediction software, SOCS3 was found to be a conserved target of miR-203. Further investigation revealed that SOCS3 was strongly expressed in the keratinocytes of healthy skin, however, it was suppressed in the epidermis of psoriatic

lesions. As constitutive activation of STAT3 in the keratinocytes of transgenic mice leads to the development of psoriasis and SOCS3 deficiency leads to the sustained activation of STAT3 in response to IL-6, it is suggested that the suppression of SOCS3 in keratinocytes may lead to sustained STAT3 activation and thus the development of psoriatic plaques. These findings reveal a new level of molecular control of immune functions that may become important targets for therapy.

miR-19a is transcribed from the oncogenic miR-17-92 cluster, implicated in B cell lymphoma and breast cancer [145]. This miRNA cluster is noted to promote proliferation, inhibit apoptosis and induce tumour angiogenesis. It is also necessary for the development of B cells. Little is known about the specific role of miR-19a, however, SOCS1 has a highly conserved consensus binding site for this miRNA in its 3'UTR and miR-19a has been shown to down regulate SOCS1 expression [146]. More importantly, miR-19a is reported to be induced by IFN- α , suggesting it may play a role in the regulation of the JAK-STAT pathway and potentially enhance, or contribute to, the role of SOCS proteins [149]. SOCS proteins and miRNAs are examples of regulators of biological processes but their mechanisms of control and their target genes differ. SOCS proteins are specific in that they primarily regulate the JAK-STAT pathway, whereas microRNAs have a broader function in regulating hundreds of target mRNAs. Even though SOCS1 and SOCS3 are important, established regulators of the JAK-STAT pathway, their own regulation is not well defined. It is possible therefore, that miRNAs regulate components of the JAK-STAT pathway, including SOCS proteins.

Evidence of the importance of regulating the JAK-STAT pathway is supported by the endogenous control mechanisms employed and the diverse hematologic malignancies that result from disrupted regulation. Negative regulation of signal transduction is necessary for an appropriate cellular and physiological response to cytokine stimulation. Since many inflammatory diseases and cancers stem from uncontrolled JAK-STAT signalling, we set out to investigate if other genes involved in JAK-STAT signal transduction had complementary sequences to miR-19a, in anticipation of identifying an alternative novel regulator of this pathway. Furthermore, we set out to determine whether the regulation of JAK-STAT pathway proteins, by miR-19a, could affect downstream biological processes of this signalling cascade.

3.2 Hypothesis

We proposed that miR-19a may play a functional role in the regulation of JAK-STAT signalling by targeting multiple components and silencing their expression. We also postulated that this might alter signal transduction within the pathway.

3.3 Aims

1. To identify genes in the JAK-STAT pathway that contained potential target sequences for miR-19a
2. Validate these sequences by transfection of a miRNA mimic and detection by qRT-PCR
3. To determine the effect on JAK-STAT signalling.

3.4 Results

3.4.1 Prediction of miR-19a targets in components of the JAK-STAT pathway

A combination of KEGG database and InnateDB database identified 158 proteins in the JAK-STAT pathway, while TargetScan predicted 938 target genes for miR-19a. By cross referencing these results, 12 protein coding genes associated with the JAK-STAT pathway were identified to contain miR-19a target sequences in their 3'UTR (Table 3.1). Thus 7.1% of the genes in the JAK-STAT pathway (compared to 3.7% of the human genome) contains miR-19a target proteins ($p=0.019$). In comparison, no members of the 102 Toll-like receptor pathway contain target sequences for miR-19a, suggesting for the first time that miR-19a has significant specificity for regulation of the JAK-STAT pathway. TargetScan revealed that all 12 JAK-STAT genes had 7-8 base predicted sequences that matched miR-19a in their 3'UTRs (Table 3.2A). As our primary interest is in regulation, we chose to further investigate the regulatory genes SOCS1, SOCS3, SOCS5 and Cul5. We found that the miR-19a target was conserved in each of these genes across several species, including chimpanzee (Ptr), rhesus (Mml), dog (Cfa), mouse (Mmu) and horse (Eca) (Table 3.2B), strongly indicating a precise and important functional role for miR-19a in the control of JAK-STAT pathway components.

SOCS1	SPRY3	CCND1
113		

SOCS3	STAM2	CCND2
SOCS5	STAT2	CNTFR
SPRED2	PIK3R3	CUL5

Table 3.1: JAK-STAT proteins containing miR-19a target sites

12 protein coding genes identified to be involved in JAK-STAT signalling and contain miR-19a targets sites in their 3’UTRs.

A

Position	3'UTR	Target binding sequence
305-311	SOCS1	5'...AUC <u>UUUGCACA</u> ...
1560-1566	SOCS3	5'...AAC <u>UUUGCACA</u> ...
1212-1218	SOCS5	5'...CAU <u>UUUGCACU</u> ...
1585-1591	SPRED2	5'.....UAU <u>UGCACA</u> ...
2934-2940	SPRY	5'...GUA <u>UUGCACA</u> ...
1333-1339	STAM2	5'....AAAU <u>UUGCAC-A</u> ...
693-699	STAT2	5'...AGC <u>UUUGCACA</u> ...
94-100	PIK	5'...UGU <u>UUUGCACA</u> ...
1778-1784	CCND1	5'....CAA <u>UUUGCACA</u> ...
1477-1483	CCND2	5'...UUU <u>UUUGCACA</u> ...
81-87	CNTFR	5'...UAU <u>UUUGCACA</u> ...
189-195	CUL5	5'...AUG <u>UUGCACA</u> ...
364-370	CUL5	5'...UAC <u>UUGCACA</u> ...
	hsa-miR-19a	3'.....AAACGUGU...

B

Species	Position in 3'UTR	Target binding sequence
Mml:	305-311 of SOCS1 3'UTR	UAUC- <u>UUUGCACA</u> ...
Ptr:	305-311 of SOCS1 3'UTR	UAUC- <u>UUUGCACA</u> ...
Cfa:	305-311 of SOCS1 3'UTR	UAUC- <u>UUUGCACA</u> ...
Mmu:	305-311 of SOCS1 3'UTR	UAUC- <u>UUUGCACA</u> ...
Eca:	305-311 of SOCS1 3'UTR	UAUC- <u>UUUGCACA</u> ...
Mml:	1560-1566 of SOCS3 3'UTR	CAAAC <u>UUUGCACA</u> ...
Ptr:	1560-1566 of SOCS3 3'UTR	CAAAC <u>UUUGCACA</u> ...
Cfa:	1560-1566 of SOCS3 3'UTR	CAAAC <u>UUUGCACA</u> ...
Mmu:	1560-1566 of SOCS3 3'UTR	AGAC <u>UUUGCACA</u> ...
Eca:	1560-1566 of SOCS3 3'UTR	CAAAC <u>UUUGCACA</u> ...
Mml:	1212-1218 of SOCS5 3'UTR	ACAU- <u>UUUGCAC</u> ...
Ptr:	1212-1218 of SOCS5 3'UTR	ACAU- <u>UUUGCAC</u> ...
Cfa:	1212-1218 of SOCS5 3'UTR	ACAU- <u>UUUGCAC</u> ...
Mmu:	1212-1218 of SOCS5 3'UTR	GCCA- <u>UUUGCAC</u> ...
Eca:	1212-1218 of SOCS5 3'UTR	ACGU- <u>UUUGCAC</u> ...
Mml:	189-195 of CUL5 3'UTR	UCAUG <u>UUGCA</u> -CA...
Ptr:	189-195 of CUL5 3'UTR	UCAUG <u>UUGCA</u> -CA...
Cfa:	189-195 of CUL5 3'UTR	UCAUG <u>UUGCA</u> -CA...
Mmu:	189-195 of CUL5 3'UTR	UCAUG <u>UUGCA</u> -CA...
Eca:	189-195 of CUL5 3'UTR	UCAUG <u>UUGCA</u> -CA...
Mml:	364-370 of CUL5 3'UTR	UGUAC <u>UUGCACA</u> ...
Ptr:	364-370 of CUL5 3'UTR	UGUAC <u>UUGCACA</u> ...
Cfa:	364-370 of CUL5 3'UTR	UGUAC <u>UUGCACA</u> ...
Mmu:	364-370 of CUL5 3'UTR	UAUAC <u>UUGCACA</u> ...
Eca:	364-370 of CUL5 3'UTR	UGUAA <u>UUGCACA</u> ...

Table 3.2: Components of the JAK-STAT pathway contain miR-19a target sites in their 3'UTRs

(A) Sequence alignment of miR-19a and its target sites in the 3'UTRs of all 12 genes identified. (B) miR-19a binding sites in SOCS1, SOCS3, SOCS5 and Cul5 are conserved among rhesus macaque (Mml), chimpanzee (Ptr), dog (Cfa), mouse (Mmu) and horse (Eca).

Preliminary data (P): measuring endogenous miR-19a levels

miR-19a expression is reported to be induced following stimulation with IFN- α for 4h, in the Huh7 cell line [149]. To confirm this we measured miR-19a levels in Huh7 cells following stimulation with IFN- α over 4, 8, 14 and 24h by qRT-PCR. We observed no induction of endogenous miR-19a in response to IFN- α at 8, 14 and 24h. A 0.2 fold induction was detected at 4h, however, this level was inadequate to carry out functional studies on the miR-19a and so next investigated the effect of precursor miRNAs, which mimic endogenous mature miRNA and are routinely used in miRNA studies.

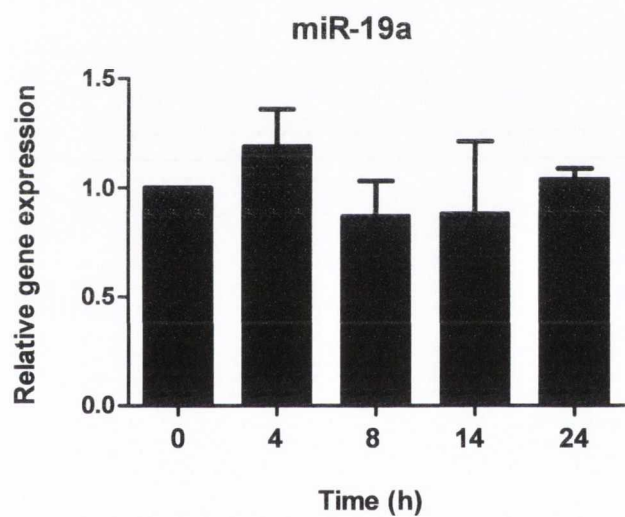


Figure P3.1: miR-19a levels in Huh7 cells

qRT-PCR analysis of miR-19a expression in Huh7 cells following stimulation with IFN- α over a time course of 4, 8, 14 and 24h. Mean (SD) fold changes are shown for each stimulation relative to the unstimulated sample (0). Results shown are the mean of 3 independent experiments.

3.4.2 Expression of SOCS1 and SOCS3 in human embryonic kidney (HEK)-293T cell line following IFN- α stimulation

HEK293T cells are highly transfectable and are regularly used for miRNA mimic studies, so we optimised the transfection of the miR-19a mimic, pre-miR-19a, in these cells. Firstly, to ensure that SOCS were expressed in this cell line, we treated them with 1000IU of IFN- α over a time course of 0.5, 1 and 2h before measuring SOCS1 and SOCS3 mRNA expression. Peak induction of SOCS3 is at 1h where an 8-fold increase in mRNA expression can be seen. SOCS1 was not induced in these cells in response to IFN- α stimulation (Figure 3.2).

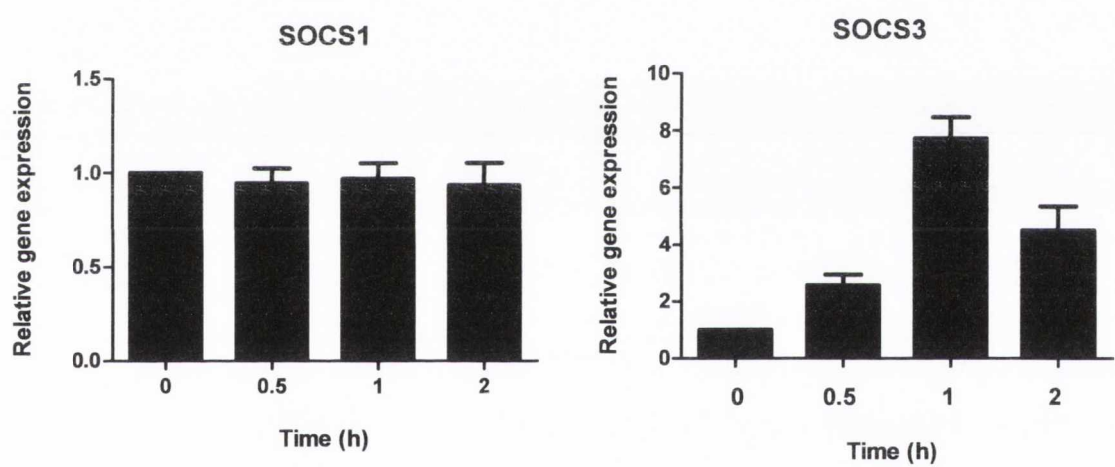


Figure 3.2: SOCS mRNA expression in HEK293T cells following IFN- α treatment

qRT-PCR analysis of SOCS1 and SOCS3 mRNA expression in HEK293T cells following stimulation with IFN- α over a time course of 0.5, 1, and 2h. Mean (SD) fold changes are shown for each stimulation relative to the unstimulated sample (0). Results shown are the mean of 3 independent experiments.

3.4.3 Optimisation and validation of miR-19a targets, SOCS1 and SOCS3, in the HEK293T cell line

Having confirmed that SOCS are expressed in HEK293T cells, pre-miR-19a was transfected and the mRNA expression of SOCS1 and SOCS3 was analysed. A mature miRNA negative control (MMNC) was also transfected into the cells. miR-19a was measured by qRT-PCR to ensure transfection was successful (Figure 3.3). Concentrations of 5 and 7.5nM of pre-miR-19a were sufficient to silence SOCS1 and SOCS3 mRNA expression (Figure 3.4). 10nM had no effect on SOCS1 or SOCS3, perhaps indicating saturation of the mimic and possible damage to the cells. Our findings validate miR-19a as a regulator of SOCS1 and SOCS3 expression in HEK293T cells and indicate that the optimal concentration of pre-miR-19a is 5nM as this resulted in the greatest down-regulation of SOCS1 and SOCS3. Higher concentrations of the mimic appear to be having less or no effect on SOCS expression. The purpose of these experiments were to establish the system, optimise the concentration of miRNA mimic and the duration of transfection, for this reason the experiments were carried out once only.

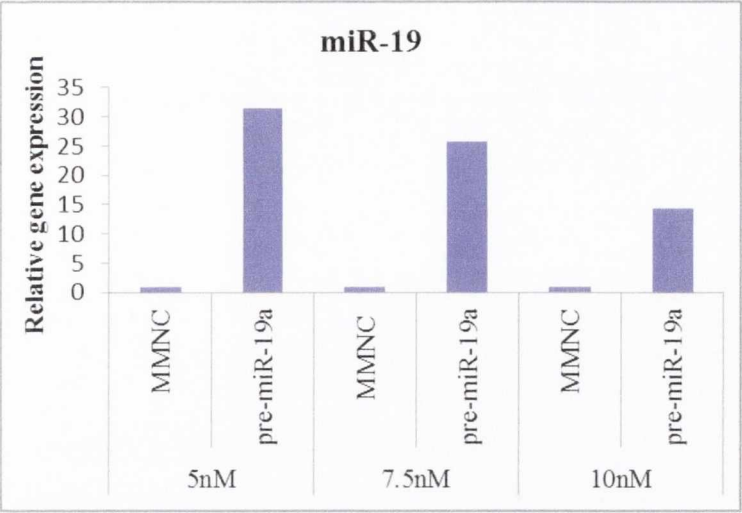


Figure 3.3: HEK293T cells expressing miR-19a

qRT-PCR confirmation of miR-19a in HEK293T cells transfected with increasing concentrations of pre-miR-19a and MMNC for 24h. Fold changes shown are relative to the MMNC (n=1).

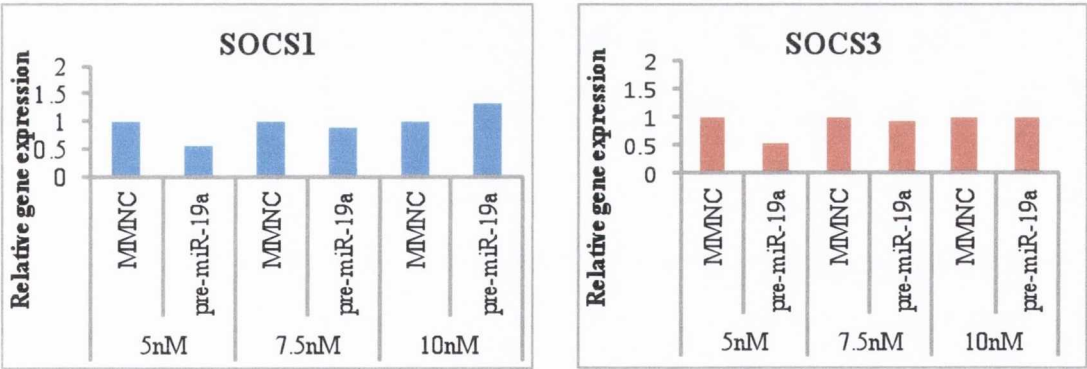


Figure 3.4: SOCS mRNA levels in HEK293T cells following transfection with miR-19a mimic

qRT-PCR analysis of SOCS1 and SOCS3 mRNA expression in HEK293T cells transfected with increasing concentrations (5, 7.5, 10nM) of pre-miR-19a and the control, MMNC, for 24h. Fold changes shown are relative to the MMNC (n=1).

3.4.4 Expression of SOCS1 and SOCS3 in the hepatocyte cell line, Huh7, following IFN- α stimulation

The liver is a unique immunological site and pro-inflammatory cytokines induced during bacterial infection can activate hepatocytes to produce acute phase proteins, such as C-reactive protein and mannose-binding lectin. These acute phase proteins act as opsonins and also activate complement. Additionally, in order to avoid over-reactions due to the continuous exposure of food and microbial antigens, the liver has developed a tolerogenic immune environment. This is achieved by specialized resident cells, including DCs and hepatocytes, expressing anti-inflammatory mediators and inhibitory cell surface ligands for T cell activation [201].

Huh7 cells are hepatocytes and because the liver plays a significant role in inflammation, and SOCS proteins regulate inflammation, we decided to investigate their expression in this cell line. SOCS1 and SOCS3 mRNA levels were measured following IFN- α stimulation over separate time courses of 0.5, 1, 2, 4 and 8, 14 and 24h. Results show that SOCS1 and SOCS3 are highly inducible following IFN- α stimulation, with gene expression peaking at 4h and then decreasing significantly by 8h in both SOCS1 and SOCS3, reflecting the short half-life of SOCS proteins (Figure 3.5).

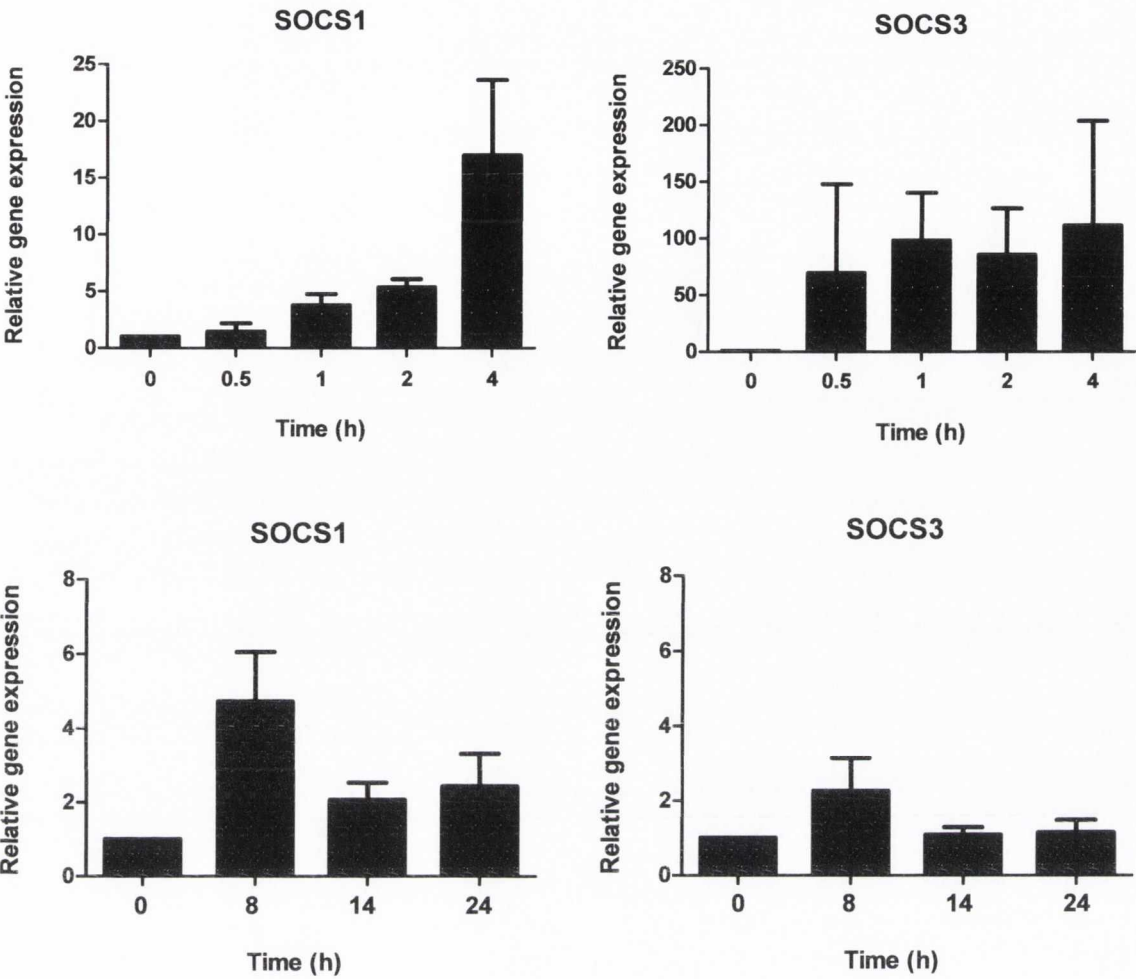


Figure 3.5: SOCS1 and SOCS3 expression following IFN- α stimulation in Huh7 cells

qRT-PCR analysis of SOCS1 and SOCS3 relative mRNA expression in Huh7 cells following stimulation with 1000U of IFN- α over a time course of 0.5, 1, 2, 4 and 8, 14 and 24h (n=3). Mean (SD) fold changes are shown for each stimulation relative to the unstimulated sample (0). Results shown are the mean of 3 independent experiments.

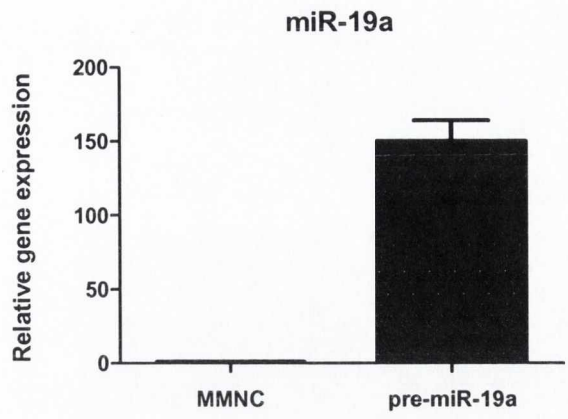
3.4.5 Validation of miR-19a targets in the Huh7 cell line

Having identified a miR-19a binding site in 4 known regulators of the JAK-STAT pathway (SOCS1, SOCS3, SOCS5 and Cul5), we were interested in investigating the functional effect of miR-19a on these genes. Huh7 hepatocytes were transfected with pre-miR-19a and expression of SOCS1, SOCS3, SOCS5 and Cul5 mRNA was measured by qRT-PCR. Upon expression of miR-19a (Fig. 3.6A), there was significant down regulation of SOCS3 ($p=0.0010$) when compared to control cells.

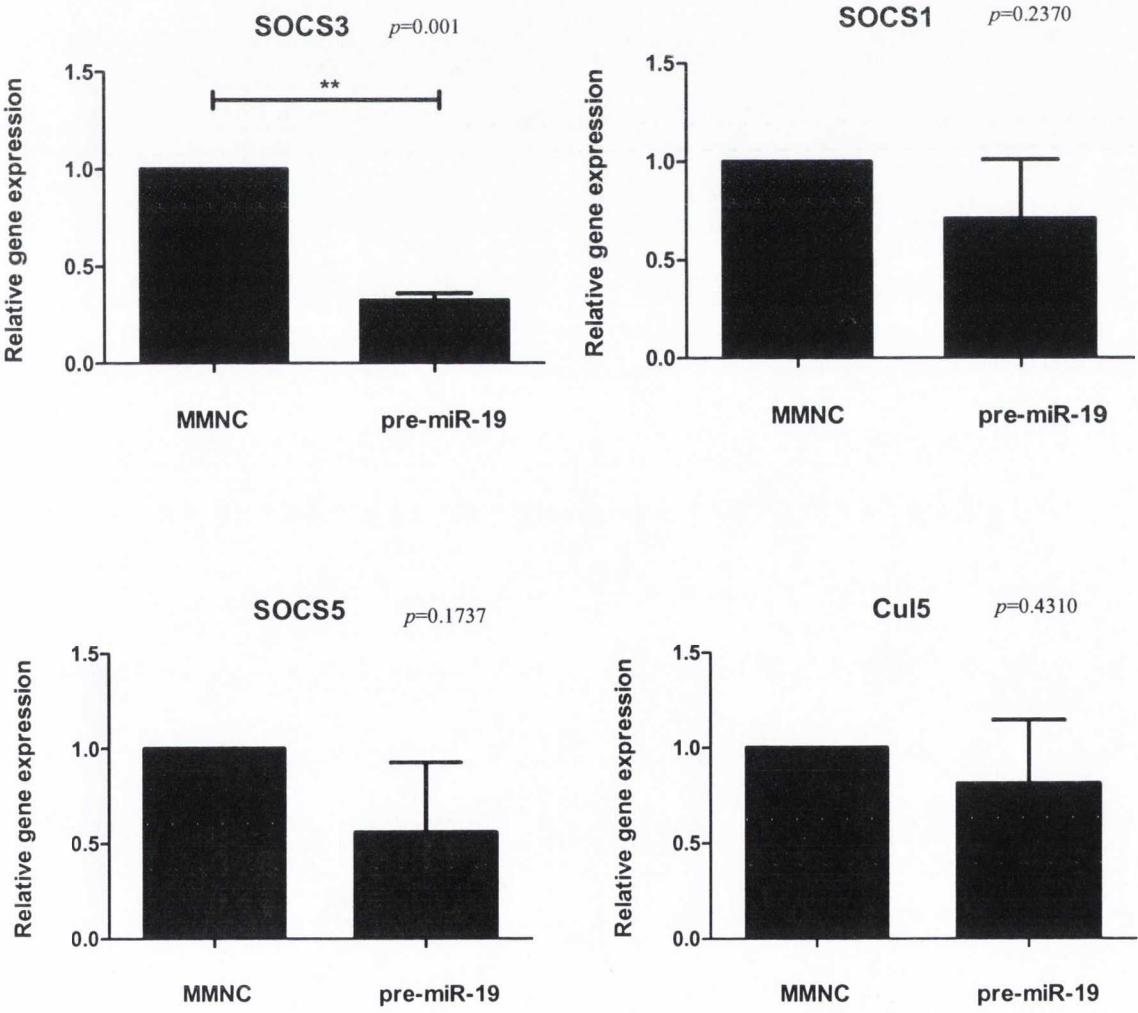
Expression of SOCS1 ($p=0.2370$), SOCS5 ($p=0.1737$) and Cul5 ($p=0.4310$) was influenced by miR-19a, but not significantly (Fig. 3.6B). SOCS2 mRNA, which does not contain a 3'UTR target sequence for miR-19a, did not decrease in the presence of miR-19a, demonstrating specificity of miR-19a for target interaction. These results reveal that the expression of the JAK-STAT pathway inhibitor, SOCS3, is significantly regulated by miR-19a.

As SOCS3 mRNA expression was significantly down-regulated by miR-19a, we set out to further investigate this finding. Initially, we analysed the effect of miR-19a upon SOCS3 3'UTR, using a luciferase reporter assay. A construct containing the 3'UTR of SOCS3 was transfected into 293T cells together with increasing concentrations of a miR-19 antagomir. We observed marked increase in luciferase activity in the presence of the miR-19 antagomir, while mutation of the predicted 3'UTR-binding site abrogated this effect (Fig. 3.6C, carried out by Dr. Clair McCoy). These results confirm SOCS3 as a functional target of miR-19a, which in turn may have implications for the regulation of the pathways controlled by SOCS3.

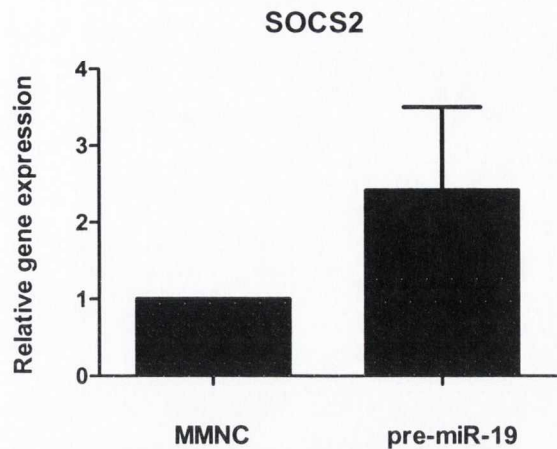
A



B



B



C

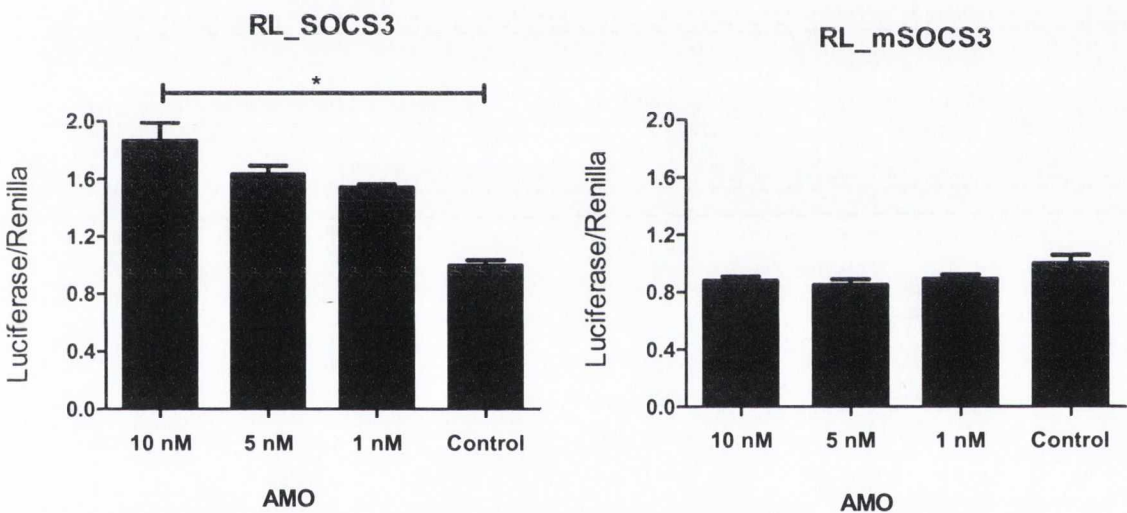


Figure 3.6: miR-19a silences SOCS3 of the JAK-STAT pathway

qRT-PCR analysis of (A) miR-19a (n=3) and (B) SOCS1, SOCS3, SOCS5, Cul5 and SOCS2 (n=3) mRNA in Huh7 cells transfected with pre-miR-19a compared to cells transfected with MMNC. Mean $\pm$ S.D of the relative gene expression was compared to the MMNC. *P* values were calculated using paired t-tests. Statistical significance is denoted as: **P*<0.05, ***P*<0.01. Results shown are the mean of 3 independent experiments. (C) Cells were transfected with pRL_SOCS3 3'UTR and pRL_mSOCS3 (mutated) 3'UTR with varying concentrations (0, 1, 5 and 10nM) of miR-19 antagomir (AMO-miR-19). Cells were lysed and analysed for *Renilla* expression. Experiments were performed in triplicate and graph is representative of one independent experiment.

3.4.6 miR-19a reduces SOCS3 protein expression and enhances IFN- α and IL-6-induced pSTAT3

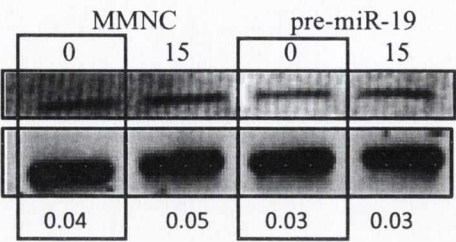
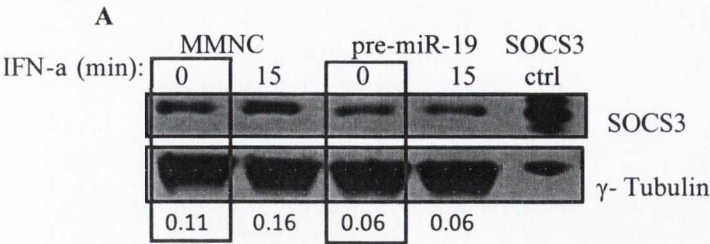
Since miR-19a reduced SOCS3 mRNA and luciferase activity, we next examined protein levels to further confirm this down-regulation. Upon expression of miR-19a, in Huh7 hepatocytes, SOCS3 protein levels decreased over 24 and 48h, compared to MMNC (Figure 3.7A). This decrease was calculated using densitometry software on the LiCor Odyssey imaging system. The band intensity of the gene of interest was normalised to the band intensity of γ -Tubulin.

Next we set out to investigate if silencing SOCS3 had a functional effect on the signal transduction of IFN- α and IL-6, known to be regulated by SOCS3. We discovered that expression of miR-19a enhanced IFN- α and IL-6-induced pSTAT3, compared to controls, following stimulation for 15min over 24 and 48h, and additionally at 30min over 24h (Figure 3.7B). This indicates that miR-19a may functionally regulate SOCS3 expression in order to promote STAT3 mediated signal transduction of IFN- α and IL-6. Furthermore, these results were consistent over 72h (Figure 3.7C). As the blots shown are from one experiment only, the average densitometric quantification of SOCS3 and pSTAT3 protein was calculated over the 3 experiments carried out and a graph of the mean values is shown in Figure 3.8.

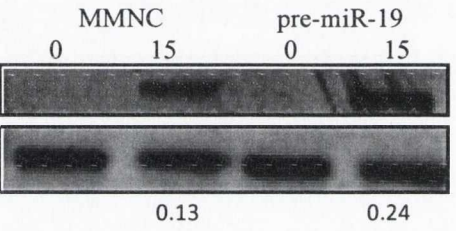
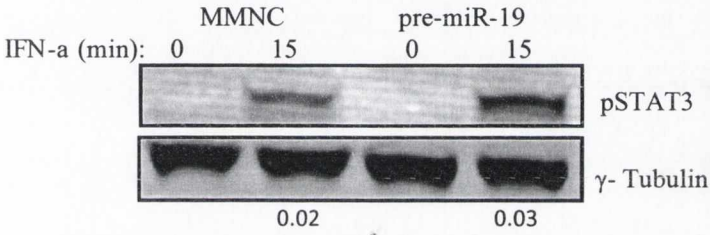
The effects of miR-19a mimic on SOCS3 expression, and IFN- α and IL-6 stimulation on pSTAT3 levels was also examined in HEK293T cells. While a decrease in SOCS3 was observed following transfection with miR-19a mimic over 24h, no increase in pSTAT3 was detected following stimulation with IL-6 or IFN- α . Moreover, no further regulation was observed at later time points of 48 and 72h, possibly indicating cell type specific regulation of cytokine signalling by miR-19a (Figure 3.9).

24h:

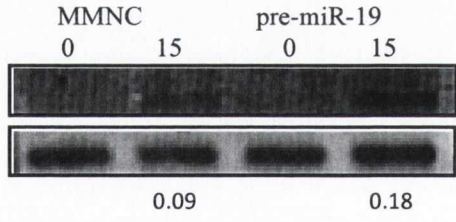
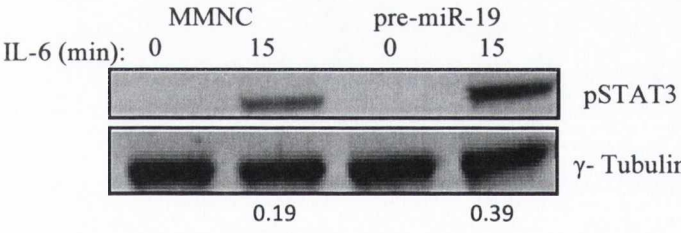
48h:



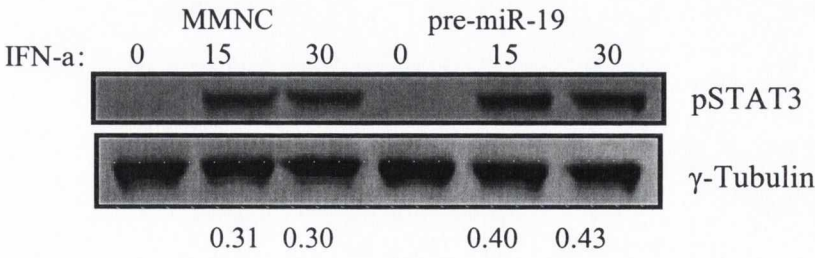
B (i)



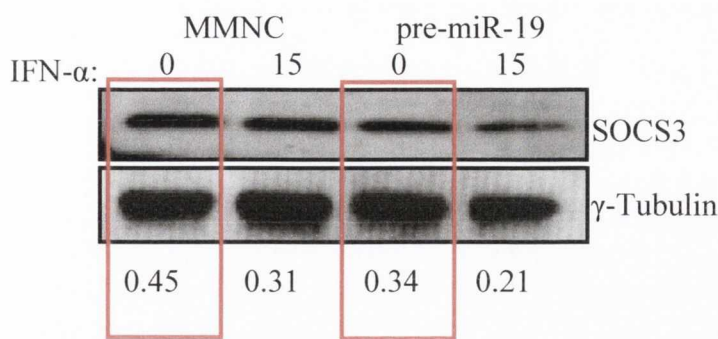
(ii)



(iii)



C: 72h



C (i)

(ii)

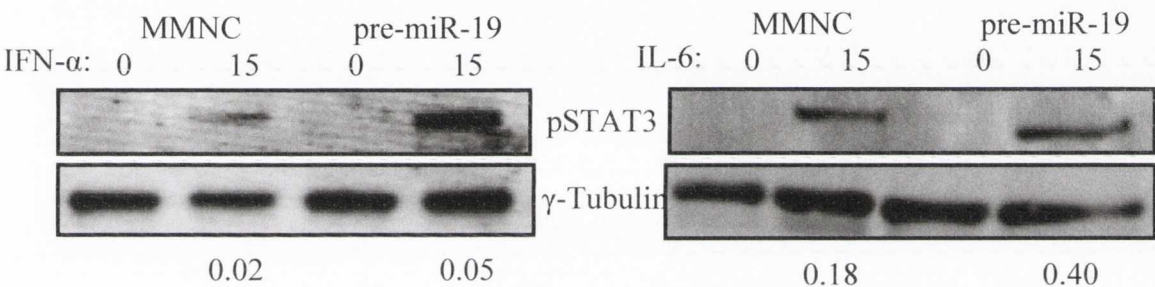
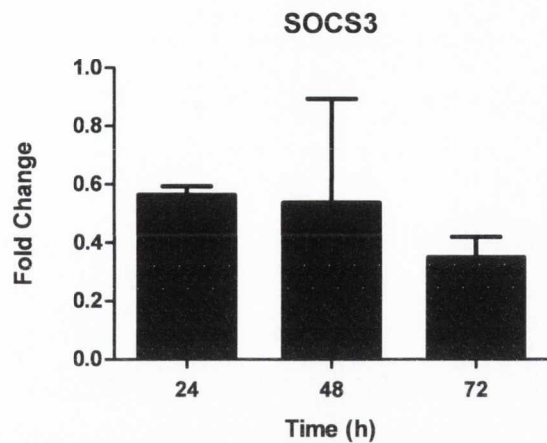


Figure 3.7: Decreased SOCS3 and increased pSTAT3 in the presence of miR-19a

(A) SOCS3 protein basal levels were reduced in Huh7s transfected with pre-miR-19a (highlighted), while (B) pSTAT3 was increased following stimulation with IFN- α (i) and IL-6 (ii) for 15min, over 24 (left panel) and 48h (right panel). pSTAT3 examined at 30min (iii), was consistent with results obtained at 15min (3B). (C) SOCS3 protein was decreased in Huh7s transfected with pre-miR-19a (highlighted), while pSTAT3 was increased following stimulation with IFN- α (i) and IL-6 (ii) for 15min over 72h. Values below blots represent the ratio of the gene of interest to γ -Tubulin. Protein lysates were analysed by immunoblotting for SOCS3, pSTAT3 and γ -Tubulin using specific antibodies. Experiments were carried out 3 times independently; blots are representative of three independent experiments.

A



B

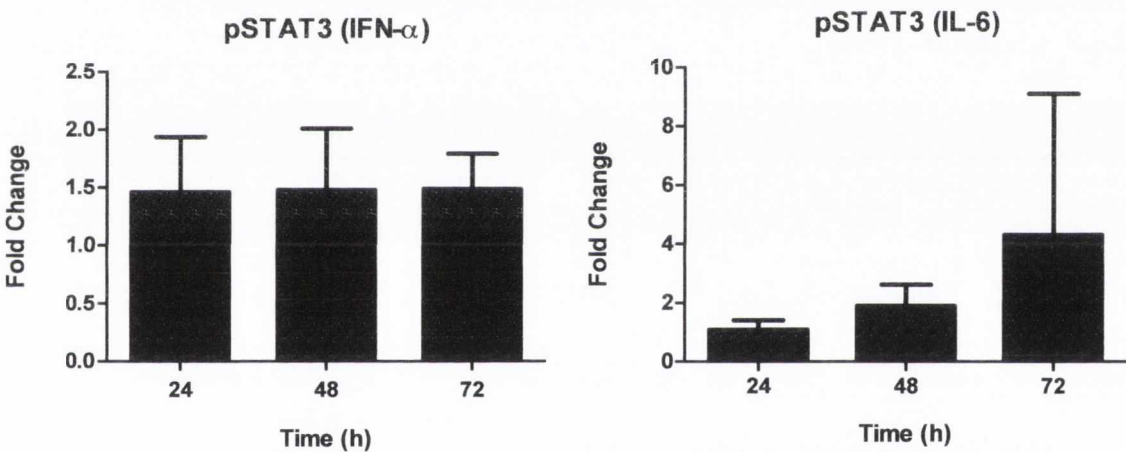
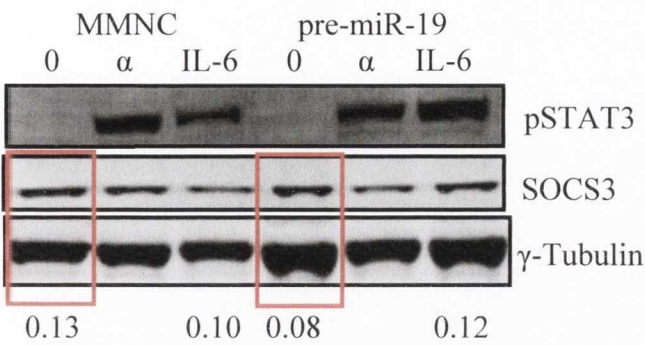


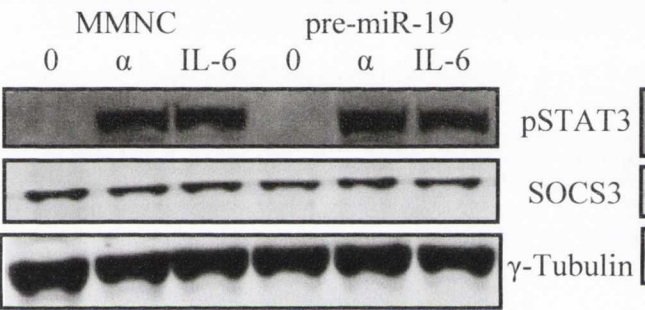
Figure 3.8: Average quantization of SOCS3, and IFN- α and IL-6 induced pSTAT3.

(A) The mean $\pm$ SD quantization of SOCS3 protein levels following transfection with miR-19a mimic over 24, 48 and 72h (n=3). (B) The mean $\pm$ SD quantization of IFN- α and IL-6-induced pSTAT3, following transfection with miR-19a mimic over 24, 48 and 72h (n=3).

D: 24h



48h:



72h:

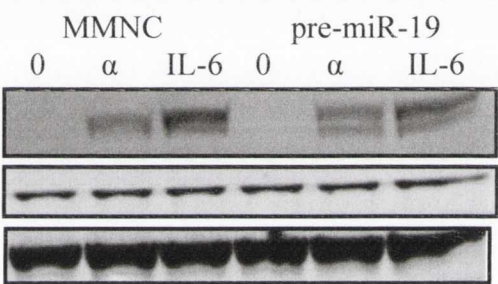


Figure 3.9: Decreased SOCS3 does not increase pSTAT3 expression in HEK293T cells

SOCS3 protein basal levels were reduced in HEK293Ts transfected with pre-miR-19a for 24h (highlighted), while pSTAT3 levels were unchanged following stimulation with IFN- α and IL-6 for 15min. pSTAT3 levels were consistently unaffected following stimulation with IFN- α and IL-6 over 48 and 72h. Values below blots represent the ratio of the gene of interest to γ -Tubulin. Protein lysates were analysed by immunoblotting for SOCS3, pSTAT3 and γ -Tubulin using specific antibodies (n=3). Experiments were carried out 3 times independently; blots are representative of three independent experiments.

3.5 Discussion

Over the past few years, several studies have illustrated the biological relevance of miRNAs in the development of the immune system and the regulation of immune functions. miR-19a has been shown to control SOCS1 [146], a well-known negative regulator of the JAK-STAT pathway and therefore we were interested in examining the effect of miR-19a on other proteins within this pathway. Using KEGG database, InnateDB and TargetScan, we identified miR-19a sequence matches in the 3'UTRs in 12 of the 158 proteins that constitute the JAK-STAT pathway. We found that the miR-19a mimic significantly down-regulated SOCS3 expression. Expression of SOCS1, SOCS5 and Cul5 mRNA was also reduced, albeit not significantly, which may indicate that miR-19a is not the primary regulator of these genes. We observed that SOCS3 protein levels decreased in the presence of miR-19a. Furthermore, we found enhanced cytokine-induced STAT3 phosphorylation, possibly as a result of reduced SOCS3-mediated regulation of the IFN- α and IL-6 pathways. These results provide a novel functional role for miR-19a in the regulation of immune signalling and highlight it as a specific regulator of SOCS3.

miR-19a is one of six precursors transcribed as one common primary transcript from the miR-17-92 cluster located on chromosome 13 in the human genome [143]. Several studies linked its expression to cancer, including B-cell lymphoma, lung and breast cancer [202,203]. SOCS3 negatively regulates the JAK-STAT pathway by targeting the STAT receptor binding site and is vital in controlling the duration and amplitude of JAK-STAT signalling [113]. The JAK-STAT pathway is critical for effective antiviral immunity and we found that miR-19a significantly targets SOCS3 and that its presence

enhanced IFN- α and IL-6-induced pSTAT3, indicating that miR-19a may functionally contribute to the regulation of both antiviral and pro-inflammatory responses.

miR-19a is reported to be induced in response to IFN- α stimulation at 4h [149]. We have shown that peak SOCS3 induction occurs 4h post IFN- α stimulation and returns to baseline level by ~8h, in Huh7 cells. SOCS proteins have a short half-life yet the mechanism by which their turnover is controlled is poorly understood. As SOCS3 is induced by a number of cytokines (see page 54), miR-19a may in fact be a novel regulator involved in maintaining low SOCS3 levels following transient induction. Cytokine signalling (e.g. IL-6) via STAT3 can enhance SOCS3 expression in the cell, resulting in down-regulation of the JAK-STAT pathway. The induction of miR-19a may act to fine tune the levels of SOCS3, thereby insuring an efficient, controlled JAK-STAT response. Interestingly, the miR-17-92 cluster from which miR-19a is transcribed from is reported to be STAT3 dependent [204], indicating that a number of cytokines which signal via STAT3 may also up-regulate this miRNA.

Roles for STAT3 and SOCS3 in the regulation of homeostasis and inflammation have been documented. SOCS3 knockout mice die embryonically as a result of liver erythropoiesis, demonstrating a key role for SOCS3 in controlling cell homeostasis [113]. STAT3 knockout mice also die during embryogenesis however mice with STAT3-deficient macrophages and neutrophils are susceptible to endotoxin shock and demonstrate increased production of inflammatory cytokines [205]. STAT3 and SOCS3 are said to control the function and number of neutrophils in order to maintain tissue inflammation, without causing destruction [206]. G-CSF can stimulate the maturation and exit of neutrophils from the bone marrow and is often used as a treatment during chemotherapy and genetic neutrophil deficiencies. The G-CSF receptor (G-CSFR) transduces the signals from G-CSF via four tyrosine residues located in the cytoplasmic

region of the receptor, leading to the activation of STAT molecules, including STAT3. Loss of function of STAT3 or SOCS3 resulted in the accumulation of late stage neutrophils in the bone marrow and peripheral blood, indicating a role for STAT3 and SOCS3 in the regulation of granulopoiesis. Hortner *et al.*, discovered that SOCS3 binds to a specific tyrosine residue in the G-CSFR and limits the amplitude of STAT3 signalling; consequently loss of SOCS3 function resulted in an increase in G-CSFR signalling and thus neutrophil accumulation. Moreover, loss of STAT3 function prevents the induction of SOCS3 which regulates G-CSFR, also resulting in increased neutrophil numbers [207].

Furthermore, a role for SOCS3 in anti-inflammatory IL-10 signalling has been noted. IL-10 induces the expression of SOCS3, which can dampen STAT3-mediated inflammatory signalling via the IL-6 pathway, by binding to a tyrosine residue on gp130 receptor [206]. These findings highlight the specific role of SOCS3 in negatively regulating inflammatory signalling pathways and STAT3 activation, and emphasise the importance of identifying miR-19a as a regulator of this inhibitory molecule.

Interestingly, Wang *et al.*, have demonstrated a role for SOCS1 and miR-155 in innate immunity during viral infection. They show that RNA virus infection induces miR-155 expression in macrophages via the RIG-I pathway, which in turn promotes type I IFN signalling by suppressing SOCS1 expression and subsequently enhancing viral clearance [147]. Moreover Pichiorri *et al.*, reported a role for miR-19a in the regulation of SOCS1 in MM cell lines. However we did not observe a significant regulation of SOCS1 by miR-19a in Huh7 cells, which may be indicative of tissue-specific regulation and expression levels of miR-19a.

In conclusion, we have shown that miR-19a plays a specific role in the negative regulation of SOCS3, thus promoting STAT3 phosphorylation. miR-19a also influences SOCS1, SOCS5 and Cul5 expression. It is likely that miRNAs work alongside other mechanisms as an additional level of control or possibly to “fine tune” existing regulatory molecules. Our results indicate that miR-19a may enhance JAK-STAT signalling as a further layer of regulation of cytokine responses. In fact, these regulatory effects of miR-19a may provide a missing link to our understanding of immune regulation, that in the future may be harnessed to control inflammatory disorders associated with dysregulation of JAK-STAT signalling and aberrant SOCS3 expression.

Chapter 4

SOCS expression in the PBMCs of healthy and HCV infected individuals and Huh7 hepatocytes expressing HCV

CHAPTER 4: SOCS EXPRESSION IN THE PBMCs OF HEALTHY AND HCV INFECTED INDIVIDUALS AND HUH7 HEPATOCYTES EXPRESSING HCV

4.1 Introduction

Viral infection of cells induces the production of a group of proteins known as IFNs. IFN- α is a type I IFN and pleiotropic cytokine, known primarily for its ability to inhibit viral replication, but it also exhibits potent immunoregulatory and anti-proliferative properties [208]. IFN- α is synthesised in response to ds-RNA, produced during the infectious cycle of viruses. Following expression and secretion, it binds with high affinity to heterodimeric cell surface receptors and this triggers an intracellular cascade of events, via the JAK-STAT pathway. Virus-induced IFN- α results in the induction of powerful antiviral proteins, known as ISGs, which cause the destruction of mRNA and inhibit the translation of viral proteins [209]. Additionally, IFN- α can activate NK cells, which kill virus-infected cells and induce MHC class I expression in most cell types, thereby enhancing antigen presentation [12].

Type I IFNs are among the most important components of current therapy against HCV. IFN- α in combination with the antiviral agent ribavirin is the current treatment for HCV infection, however, response is successful in only ~50% of patients depending on viral genotype. HCV's capacity to interfere with the IFN- α signalling pathway is well documented and IFN- α -induced JAK-STAT signalling was shown to be inhibited in cells that express HCV proteins, and in liver biopsies of HCV patients [210]. SOCS proteins negatively regulate the JAK-STAT pathway in order to control the duration and amplitude of IFN- α signalling and maintain homeostasis in the cell. Therefore, virus-induced expression of these powerful inhibitors is an ideal instrument to

counteract the immune response to IFN- α . Thus, HCV-induced SOCS proteins have been implicated in the resistance to IFN- α .

HCV is a liver-trophic, positive-stranded RNA flavivirus that infects and replicates primarily in the hepatocytes of the liver, leading to chronic infection associated with significant liver disease, including cirrhosis and hepatocellular carcinoma [211]. However, HCV replication has also been reported to occur in extra-hepatic reservoirs, such as PBMCs [212-214]. The clinical symptoms associated with HCV involve a number of extrahepatic complications including mixed cryoglobulinemia, non-Hodgkin's lymphoma, glomerulonephritis, autoimmune disorders, neuropathy and lymphoproliferative disorders, indicating the involvement of HCV in a variety of non-hepatic pathogenic processes [212]. The ability to detect a HCV RNA minus strand is required to confirm extra-hepatic replication. Natarajan *et al.*, identified the presence of a minus strand in the PBMCs of HCV/HIV co-infected individuals. They also determined that HCV is predominantly carried on the surface of NK cells, monocytes and B cells [215]. Detection of HCV RNA negative strand has been suggested to be more often detected in the PBMCs from HCV genotype 1 infected individuals. Patients with detectable HCV RNA minus-strand in PBMCs had lower sustained response rates to IFN treatment when compared to those without detectable minus-strand HCV RNA in PBMCs [212].

A number of studies have reported on the expression of SOCS1 and SOCS3 during HCV infection in relation to inhibition of the JAK-STAT signalling pathway and resistance to IFN- α therapy. Bode *et al.*, reported that overexpression of HCV's core protein, in HepG2 cells, inhibited IFN- α -induced tyrosine phosphorylation of STAT1 with a concurrent increase in SOCS3 mRNA expression, which is thought to antagonise IFN signalling by negatively regulating the JAK-STAT pathway [168]. Vlotides *et al.*,

demonstrated that over-expression of SOCS1 and SOCS3 in HepG2 cells is associated with a decrease in STAT1 and STAT3 phosphorylation and thus antiviral ISG production, and suggested this to be responsible for conferring IFN- α resistance during antiviral treatment for HCV [180]. Yao *et al.*, have shown that SOCS1 up-regulation and STAT1 down-regulation suppresses T cell activation, with the opposite pattern occurring in B cell activation, in response to HCV's core protein [216,217]. Huang *et al.*, have found similar results in chimpanzees; a defective hepatic response to IFN- α , most likely mediated through observed elevated SOCS3 expression [181].

Non-response to IFN- α therapy has also been linked to increased hepatic SOCS expression in HCV patients. Persico *et al.*, and Walsh *et al.*, reported that overexpression of SOCS3 in liver tissue is associated with a poorer treatment outcome in patients with HCV genotype 1, and that SOCS3 levels are higher in non-responders to therapy than responders, regardless of genotype [182,218]. Results from Kim *et al.*, indicate that hepatic SOCS3 levels are a baseline predictor of antiviral response.

In contrast, SOCS1, but not SOCS3 transcripts were reported to be significantly higher in the livers of chronic hepatitis C patients compared to controls [184] and SOCS3 has been found to inhibit HCV replication in an mTOR dependent manner [183]. Conflicting results in the field of SOCS and HCV infection have made it difficult to define their specific roles and expression patterns, however, it is clear that HCV is regulating their expression.

A major obstacle impeding HCV research was the absence of appropriate *in vitro* replication systems and practical infectious small animal models; however, significant progress has been made with the use of HCC-derived cell lines and immortalised hepatocytes. In 2003, Wakita and colleagues cloned a HCV consensus genotype 2a

genome from a Japanese Fulminant Hepatitis (JFH-1) patient, which was subsequently found to produce infectious HCV in Huh7 cell cultures [219]. Huh7 is a hepatocyte derived cellular carcinoma cell line which can be used to study HCV. The Huh7 cells used in these experiments stably overexpress T7 polymerase. The HCV cDNA construct, under the control of a T7 promoter region, expresses HCV's structural and non-structural proteins when transfected into these cells, enabling us to determine the effects of HCV proteins on SOCS expression.

SOCS1 and SOCS3 are likely targets for viral immune evasion and it is clear they have a significant part to play during HCV infection, but further investigation is required to adequately define their roles. Although results have shown that HCV is present in PBMCs, few have studied the affect it has on SOCS expression. Additionally, *in vitro* models are lacking and Huh7 cells transfected with a cDNA construct containing HCV proteins provides an ideal model to examine the effect the virus has on SOCS expression in hepatocyte cells.

4.2 Hypothesis

We proposed that SOCS1 and SOCS3 basal expression would be elevated in HCV+ PBMCs. In response to IFN- α , we postulated that SOCS induction would be inhibited as a consequence of elevated basal levels negatively regulating the JAK-STAT signalling pathway. We also sought to determine the effect of HCV on SOCS1 and SOCS3 expression in the hepatocyte cell line, Huh7.

4.3 Aims

1. Investigate the basal expression of SOCS1 and SOCS3 in healthy and HCV+ PBMCs
2. Determine whether IFN- α could induce SOCS1 and SOCS3 expression and whether this pattern of expression was similar in both groups.
3. Assess the expression and induction of SOCS1 and SOCS3 in Huh7 hepatocytes transfected with HCV

4.4 Results

4.4.1 Selection of stable normaliser gene for quantitative real-time PCR (qRT-PCR)

Using geNorm analysis software, the expression of several housekeeping genes, GAPDH, β -Actin, PPIA, GUSB, RPS9 and RPS15 were examined in cDNA from a number of healthy and HCV+ PBMC samples, and from Huh7 cells. RPS15 and RPS9 demonstrated the greatest stability across all samples and so RPS15 was selected as the housekeeping gene for normalisation of the qRT-PCR data.

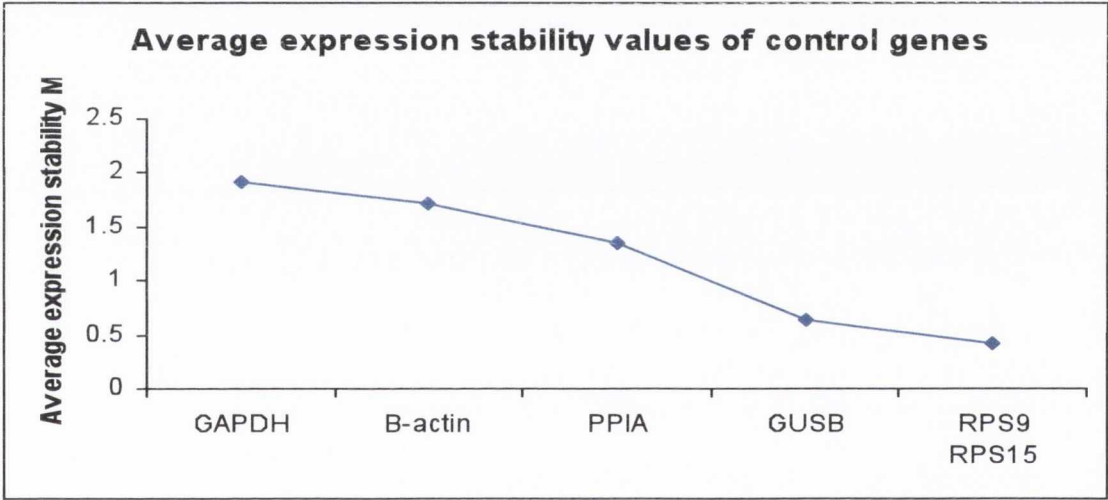


Figure 4.1: Stability of reference genes across PBMCs and Huh7 cells

The stability of a range of housekeeping genes (GAPDH, β -actin, PPIA, GUSB, RPS9 and RPS15) was tested. RPS9 and RPS15 were found to be the most stable. N=1.

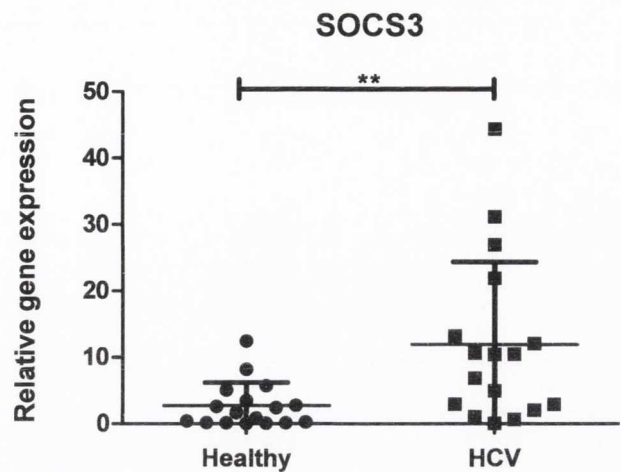
4.4.2 Differential SOCS3 gene expression levels in resting and IFN- α stimulated PBMCs from healthy and HCV+ individuals

Basal SOCS3 gene expression was detected in both healthy and HCV+ samples but was significantly upregulated in HCV+ individuals ($p=0.006$) when compared to healthy controls (Figure 4.2A). Following IFN- α stimulation, SOCS3 was induced within 0.5h in both healthy and HCV+ PBMCs and this reflects peak induction (Figure 4.2B). Although endogenous SOCS3 expression is elevated in HCV+ PBMCs, this does not appear to affect further induction of SOCS3 in response to IFN- α , which may be indicative of HCV benefitting from a high level of SOCS3 in the cell.

4.4.3 Differential SOCS1 gene expression levels in resting and IFN- α stimulated PBMCs from healthy and HCV+ individuals

The expression of SOCS1 was detected in the PBMCs from healthy and HCV infected individuals (Figure 4.3A) and a similar SOCS1 expression pattern was found in both groups. Following stimulation with 1000IU of IFN- α , SOCS1 was induced within 30mins and peaked at 2hr in both healthy and HCV+ samples (Figure 4.3B). HCV does not appear to have an effect on SOCS1 expression or induction in response to IFN- α stimulation, perhaps HCV specifically targets SOCS3 and elevated levels are sufficient to disrupt the immune response.

A) Basal SOCS3 expression in healthy and HCV+ PBMCs



B) SOCS3 induction following IFN- α stimulation in healthy and HCV+ PBMCs

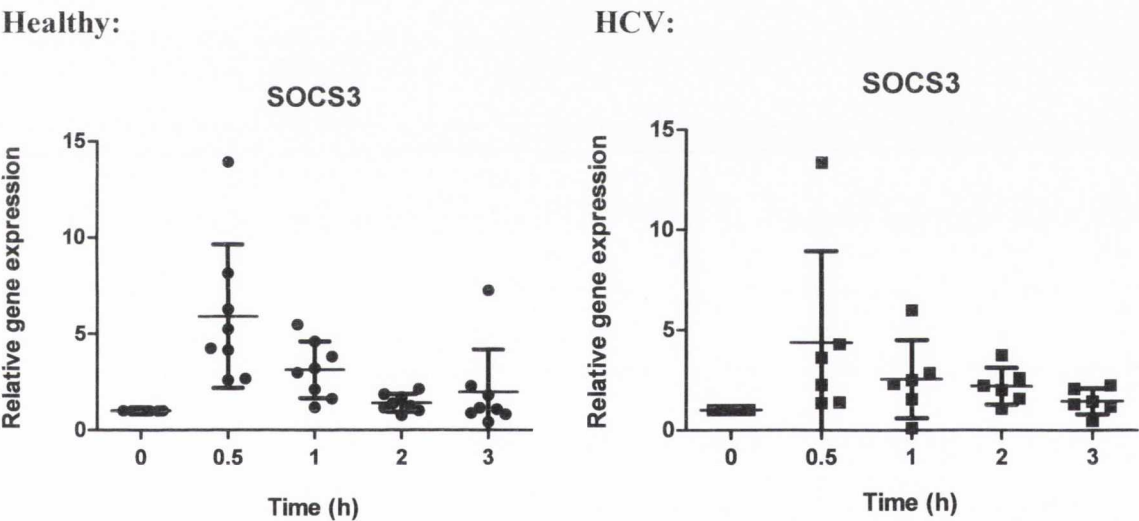
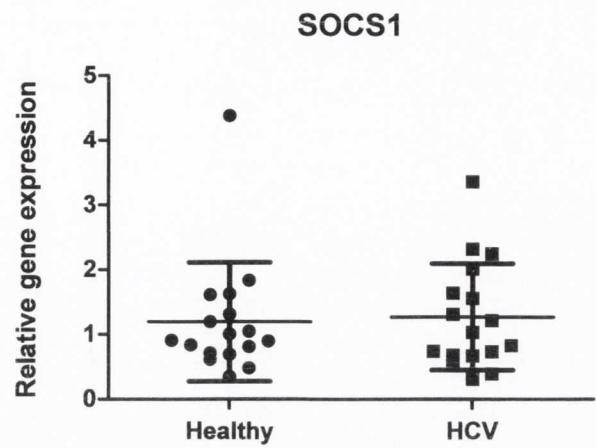


Figure 4.2: qRT-PCR analysis of SOCS3 expression and induction in PBMCs from healthy and HCV+ individuals

(A) qRT-PCR analysis of SOCS3 expression in resting PBMCs from healthy and HCV+ individuals. Mean (SD) fold changes are shown for HCV+ samples (n=17) relative to the average of the healthy controls (n=17). Non-parametric Mann-Whitney U-tests were used to test for significant differences between the two groups. Statistical significance is denoted as: **P<0.01. (B) qRT-PCR analysis of SOCS3 gene expression in healthy (n=8) and HCV+ (n=6) PBMCs following stimulation with 1000Units of IFN- α over a 4hr time course. Mean (SD) Fold changes are shown for each stimulation relative to the unstimulated sample (0).

A) Basal SOCS1 expression in healthy and HCV+ PBMCs



B) SOCS1 induction following IFN- α stimulation in healthy and HCV+ PBMCs

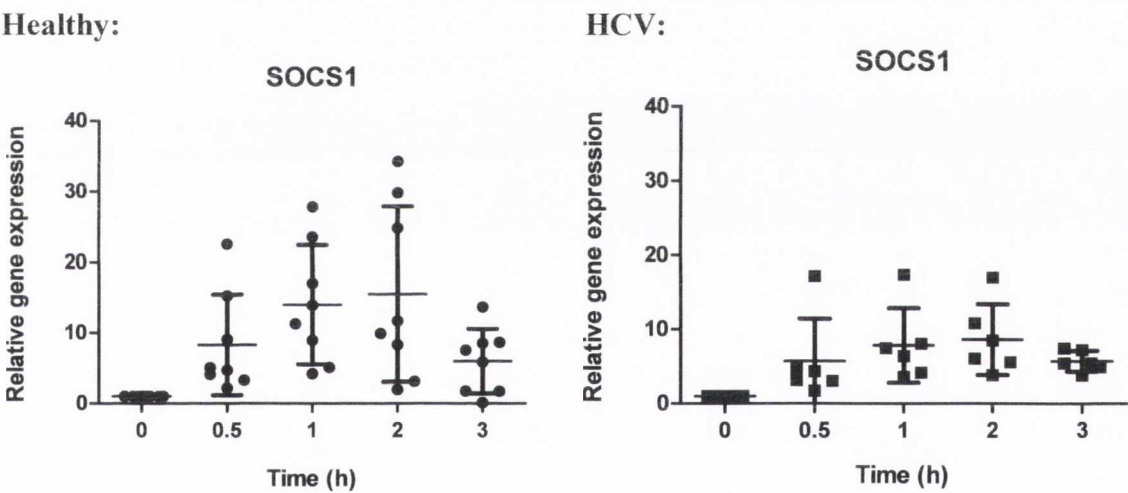


Figure 4.3: qRT-PCR analysis of SOCS1 expression and induction in PBMCs from healthy and HCV+ individuals.

(A) qRT-PCR analysis of SOCS1 expression in resting PBMCs from healthy and HCV+ individuals. Mean (SD) fold changes are shown for HCV+ samples (n=17) relative to the average of the healthy controls (n=17). (B) qRT-PCR analysis of SOCS1 gene expression in healthy (n=8) and HCV+ (n=6) PBMCs following stimulation with 1000Units of IFN- α over a 4hr time course. Mean (SD) fold changes are shown for each stimulation relative to the unstimulated sample (0).

4.4.4 SOCS1 and SOCS3 mRNA expression in response to IFN- α in a human acute monocyte leukemia cell line

Having established the pattern of SOCS1 and SOCS3 expression in HEK293T cells, Huh7 cells and PBMCs, we further confirmed SOCS responsiveness to IFN- α in the Thp-1 cell line, which is routinely used as a model of human monocytes. Cells were stimulated with 1000IU of IFN- α over a time course of 0.5, 1, 2 and 4h before measuring SOCS1 and SOCS3 mRNA levels. Peak induction of SOCS1 was observed between 1 and 3h where a 12-fold increase in mRNA expression was detected. SOCS3 induction peaked between 0.5 and 1h, with a 10-fold induction in expression compared to unstimulated cells (Figure 4.4).

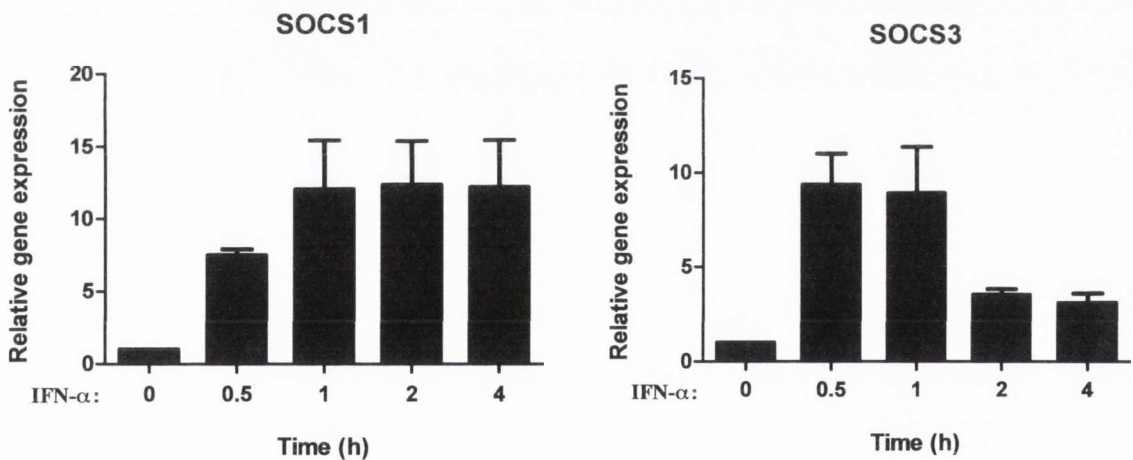


Figure 4.4: SOCS1 and SOCS3 mRNA expression in Thp-1 cells in response to IFN- α

qRT-PCR analysis of SOCS1 and SOCS3 relative mRNA expression in Thp-1 cells following stimulation with 1000IU of IFN- α over a time course of 0.5, 1, 2 and 4h (n=3). Mean (SD) fold changes are shown for each stimulation relative to the unstimulated sample (0). Results shown are the mean of 3 independent experiments.

4.4.5 SOCS1 and SOCS3 mRNA expression in resting and IFN- α stimulated Huh7 cells, transfected with HCV and EV

We previously investigated the IFN- α responsiveness of SOCS1 and SOCS3 in untransfected Huh7 cells (Figure 3.7). To determine if HCV had an effect on SOCS expression in Huh7 cells, we transfected them with a pBRTM/HCV1-3011 DNA construct and an EV for 24h. Cells were then treated with IFN- α for 1 and 4h before SOCS1 and SOCS3 mRNA levels were measured by qRT-PCR.

SOCS3 basal expression in Huh7 cells transfected with HCV was significantly ($p=0.02$) elevated when compared to EV transfected cells (Figure 4.5(i)). Induction in response to IFN- α was similar in both groups (Figure 4.5(ii)). This is in concordance with our findings of increased SOCS3 levels, and induction in response to IFN- α , in HCV+ PBMCs. Again we observed that HCV induces SOCS3 expression without affecting further induction in response to IFN- α , possibly indicating that SOCS3 may have an alternative role, which may be of benefit to HCV.

SOCS1 mRNA basal expression (Figure 4.6(i)) and induction in response to IFN- α (Figure 4.6(ii)) was similar in HCV and EV transfected Huh7 cells implying that HCV had no effect on SOCS1, concurring with our results in PBMCs.

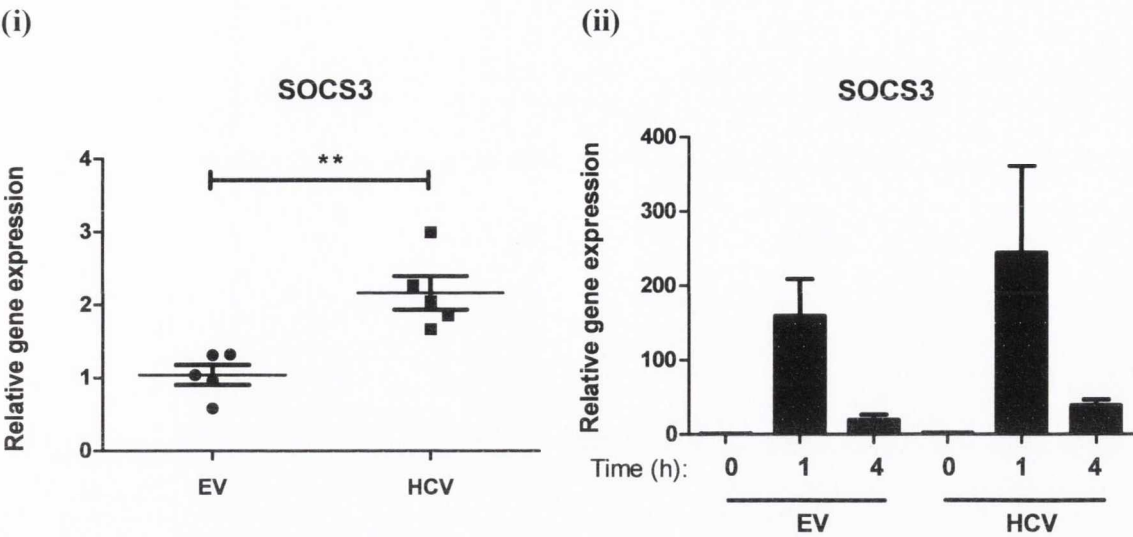


Figure 4.5: Basal and IFN- α -induced expression of SOCS3 mRNA in HCV transfected Huh7 cells

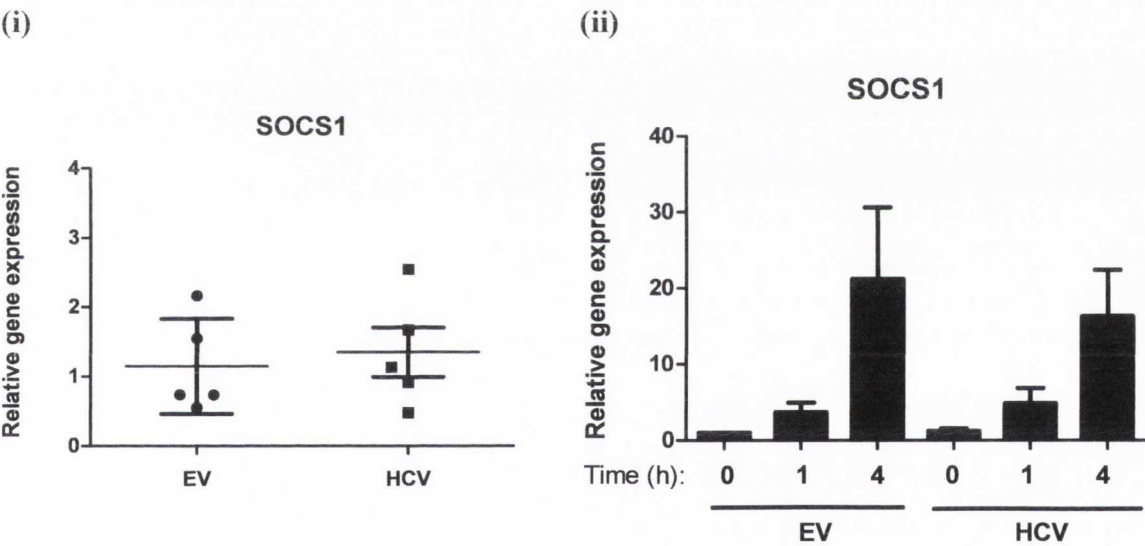


Figure 4.6: Basal and IFN- α -induced expression of SOCS1 mRNA in Huh7 cells expressing HCV

qRT-PCR analysis of SOCS3 and SOCS1 in Huh7 cells transfected with HCV and EV for 24h (n=5) and after IFN- α stimulation for 1 (n=3) and 4h (n=2), Fig. 4.5 and Fig. 4.6 respectively. Mean (SD) fold changes are relative to cells transfected with EV (i) and unstimulated EV (0) (ii). *P* values were calculated using paired t-tests. Statistical significance is denoted as: ** *P* < 0.01.

4.5 Discussion

In this study we demonstrate that basal SOCS3 mRNA expression levels are significantly upregulated in the PBMCs from HCV infected individuals, compared to healthy PBMCs, indicating that HCV is acting to induce SOCS3. Stimulation with IFN- α -induced SOCS3 expression similarly in PBMCs from both groups suggesting that this part of the JAK-STAT pathway is intact, despite the elevated SOCS3 levels. In contrast, basal levels of SOCS1 were similar in healthy and HCV+ PBMCs, implying that HCV is specifically inducing SOCS3. SOCS1 induction in response to IFN- α was also unaffected by the presence of HCV, indicating that HCV does not disrupt SOCS1 expression. Understanding SOCS expression in healthy and HCV+ PBMCs is a prerequisite to identifying the mechanisms employed by HCV to inhibit IFN- α signalling.

Improved understanding of the direct mechanism by which HCV is regulating SOCS3 expression and induction and the effects this may be having on ISG production are critical in discovering how HCV prevents effective JAK-STAT signalling and thus successful viral clearance. Bode *et al.*, demonstrated that stimulation of HepG2 cells with HCV core protein induced SOCS3 expression, however the exact mechanism was not determined [168]. Furthermore Funaoka *et al.*, generated HCV core mutants, known to be associated with poor response to IFN- α therapy and examined their effects on JAK-STAT signalling in HCV-JFH1 cell culture system. IL-6 and SOCS3 expression were significantly enhanced in the presence of core mutants, and IL-6-induced SOCS3-mediated suppression of IFN was suggested as a mechanism for IFN resistance [220]. The inducer of IL-6 remains to be clarified, however IL-6 is secreted in response to cellular steatosis and insulin resistance, both of which are observed in HCV patients.

HCV specifically targeting SOCS3 expression, while SOCS1 levels remain unchanged, is a novel finding and may be mediated by the individual transcription factors that up-regulate their expression. While SOCS1 and SOCS3 are primarily STAT-dependent genes, an online transcription factor prediction tool which allows you to search for transcription factor binding sites (<http://www.cbrc.jp/research/db/TFSEARCH.html>), has identified additional, potential transcription factors for SOCS1 and SOCS3. SOCS1 transcription is predicted to be controlled by IRF-1 and IRF-2, as well as STAT proteins, whereas SOCS3 is not. This may reveal a mechanism by which HCV achieves selective up-regulation of SOCS3, however, further experimental investigation is required to fully elucidate this phenomenon.

We also found significantly enhanced basal SOCS3 mRNA expression in Huh7 hepatocytes expressing HCV, when compared to cells transfected with EV. Induction of SOCS3 in response to IFN- α was similar in both groups. SOCS1 basal expression levels and induction following IFN- α stimulation was the same in both samples. These findings are in agreement with the observations in PBMCs.

HCV-induced end stage liver disease is the leading source of demand for liver transplantation today with up to 30% of patients having developed cirrhosis after 30 years of chronic infection [221]. The most effective treatment is IFN- α therapy, but this regimen is expensive and has variable efficacy against certain HCV genotypes and so for many HCV+ patients, liver transplantation is their only cure. Walsh *et al.*, and Persico *et al.*, have linked increased hepatic SOCS3 expression in HCV genotype 1 patients, with non-response to therapy [171,182,218]. Therefore, it is likely that during chronic infection, continuous expression of SOCS3 may prevent the upregulation of antiviral proteins required to combat HCV.

In conclusion, our findings implicate SOCS3 as an important antagonist of the *in vivo* response to IFN- α . Inducing SOCS3 expression may contribute to persistent, lifelong HCV infection in an individual, unless interrupted by IFN- α therapy. However, as increased SOCS3 levels do not hamper further up-regulation of SOCS3 via the JAK-STAT pathway, we questioned of what advantage is HCV-induced SOCS3 expression? SOCS3 has been noted in the regulation of the TNF- α /NF- κ B signalling pathway which results in the production of pro-inflammatory cytokines, implicated in chronic HCV infection. We chose to examine whether SOCS3 could play a role in the regulation of this pathway, and if HCV's interference of SOCS3 expression could impact this signalling cascade.

Chapter 5

HCV-induced SOCS3 regulates TNF- α responses

CHAPTER 5: HCV-INDUCED SOCS3 REGULATES TNF- α RESPONSES

5.1 Introduction

TNF- α is an important pro-inflammatory cytokine produced in response to viral and bacterial infection. Excess levels of TNF- α are implicated in a range of inflammatory conditions, including RA, septic shock, IBD and viral hepatitis [42,222,223]. A key role for TNF- α in the pathology associated with chronic inflammation is emphasised by successful anti-TNF- α therapeutic targeting of RA and IBD [224]. The effects of TNF- α are mediated through its receptors TNFR1 and TNFR2, leading to proliferation, inflammation and apoptosis [102]. Upon receptor binding, TNF- α signals through a cascade of cytosolic proteins, leading to I κ B degradation and the subsequent release and nuclear translocation of NF- κ B. DNA binding of NF- κ B initiates gene transcription of pro-inflammatory cytokines and chemokines such as IL-6, CXCL8, TNF- α and CXCL10 [225,226].

Tight regulation of the signal transduction pathways activated by TNF- α , is required to prevent potentially harmful effects. TNF- α signal transduction is regulated by ubiquitin mediated proteasomal degradation of RIP and TRAF2 [102]. This process is reversed by DUBs, such as A20, which remove Lys-63-linked ubiquitin chains from RIP and promotes Lys-48-linked polyubiquitination, thereby targeting it for proteasomal degradation, thus blocking the TNF- α pathway and promoting inflammatory regulation [227]. Unchecked TNF- α signalling results in chronic inflammation, cell death and tumourigenesis, as demonstrated in A20-deficient mice [48]. While ubiquitination is a critical regulatory mechanism of the TNF- α signalling cascade, it is likely that this

essential pro-inflammatory cytokine pathway is regulated by other unknown mechanisms to ensure an appropriate response to infection.

SOCS proteins are best known for their role as negative regulators of the JAK-STAT pathway; however, it is also believed that they function to regulate additional pathways such as NF- κ B and TLR. SOCS1 has been shown to bind to Mal, IRAK-1 and the p65 subunit of NF- κ B enhancing their ubiquitination and degradation, thereby inhibiting NF- κ B signalling [116,117]. Other studies have shown that SOCS3 can suppress TNF- α -induced phosphorylation of the MAPKs ERK1 and ERK2, p38 and JNK and repress TNF- α induced degradation of I κ B, in primary rat islets [118]. Furthermore, SOCS3 was shown to inhibit the transcription and activation of the NF- κ B and MAPK branch of the IL-1 signalling pathway by targeting the TRAF6/TAK1 signalling complex and specifically inhibiting the ubiquitination of TRAF6 [228]. However the effects of SOCS proteins on TNF- α signalling in immune cells remains largely unknown and in this study we propose a role for SOCS in the regulation of the TNF- α /NF- κ B signalling cascade.

HCV has the ability to escape immune responses and sustain persistent viral infection with >85% of patients progressing to chronic infection over decades [229]. The long-term hepatic effect of HCV infection is extremely variable, from minimal changes to chronic hepatitis, extensive fibrosis and cirrhosis with or without HCC [230]. Acute HCV infection is asymptomatic in 50–90% of cases [230], particularly among those individuals without co-factors such as obesity, excessive alcohol intake or HIV co-infection. Indeed, some patients experience non-specific symptoms during the acute infection period and only display symptoms during chronic infection [231]. Liver pathology in chronic HCV patients is characterised by infiltration of inflammatory cells into the portal tracts and lobules. Disease progression then leads to inflammation and

liver cell necrosis causing fibrosis [98]. Liver biopsies can be used to assess the grade of liver injury to define the extent of necro-inflammatory activity, and establish the extent of fibrosis or the presence of cirrhosis. Fibrosis is overgrowth, hardening, and/or scarring of tissues resulting from chronic inflammatory reactions induced by persistent infection and leads to connective tissue replacing normal parenchymal tissue. Chemokines cooperate with pro-fibrotic cytokines in the development of fibrosis by recruiting myofibroblasts, macrophages and other key effector cells to sites of tissue injury [232].

The progression of chronic infection is believed to be mediated by pro-inflammatory cytokines, such as IL-6, CXCL8 and TNF- α . Raised ALT levels are common and may persist for years as acute enters the chronic phase of infection, however, these levels can fluctuate and may still be within the normal range in a Hepatitis C patient [233]. Cirrhosis is a consequence of chronic disease, caused by the fibrosis replacing liver tissue. A small percentage (~5-20%) of chronically infected patients develop liver cirrhosis. Furthermore, ~5% of those with cirrhosis progress to decompensated liver disease and ~2% develop HCC [234,235].

Of particular interest is the Irish Anti-D cohort, a group of women who received HCV (genotype 1b) contaminated anti-D immunoglobulin in the 1970s. Liver biopsies carried out 17 years post HCV infection revealed that in most cases, hepatic inflammation was slight or moderate (41%), indicating that chronic HCV infection is associated with slow progression of liver disease [233]. Furthermore, ~50% of these women had some degree of fibrosis and only 2% had probable or definite cirrhosis. The mechanism by which pathology is limited during HCV infection is unknown.

Preliminary data from Dr. Nigel Stevenson showed that SOCS1 and SOCS3 were induced by TNF- α in PBMCs and regulated the NF- κ B pathway by inhibiting I κ B degradation. Furthermore, a role for SOCS proteins during HCV infection has been suggested [236-239], and having found that SOCS3 is enhanced in the PBMCs of HCV infected individuals and in Huh7 cells expressing HCV, we wondered whether HCV-induced SOCS3 could reduce pro-inflammatory cytokine induction, contributing to the mild pathology associated with HCV infection [240].

Preliminary data (P), provided by Dr. Nigel Stevenson: TNF- α signalling is regulated by SOCS1 and SOCS3 via a negative feedback loop

To investigate the regulation of TNF- α signal transduction, the effect of TNF- α on SOCS expression was examined in human PBMCs. Expression of all the SOCS genes was analysed by RT-PCR over a time-course of 0-240min, in response to TNF- α stimulation. CIS and SOCS4-SOCS7 mRNA were found to be constitutively expressed in PBMCs and were not up-regulated in response to TNF- α (Figure P5.1A). However, expression of SOCS1, SOCS2 and SOCS3 mRNA was transiently increased after stimulation with TNF- α (Figure P5.1B). SOCS1 and SOCS3 protein induction mirrored the mRNA, but SOCS2 protein was endogenously expressed and showed no regulation by TNF- α (Figure P5.1C). This immediate and transient upregulation of SOCS1 and SOCS3 suggests a novel role for these inhibitory proteins in controlling TNF- α signal transduction in immune cells.

SOCS proteins are known to regulate the JAK-STAT pathway, but only a few studies have documented their involvement in NF- κ B signalling [241-243]. Therefore, to investigate the effect of SOCS1 and SOCS3 on the TNF- α /NF- κ B pathway, SOCS1^{-/-}, SOCS3^{-/-}, SOCS1^{+/+} and SOCS3^{+/+} MEFs were treated with TNF- α for 0 and 20min and I κ B degradation was measured. I κ B degradation occurred upon TNF- α treatment in WT, SOCS1 null and SOCS3 null cells, but was partially inhibited in both SOCS1 expressing and SOCS3 expressing cells (Figure P5.2A). SOCS1 and SOCS3 protein levels were confirmed by immunoblotting (Figure P5.2B).

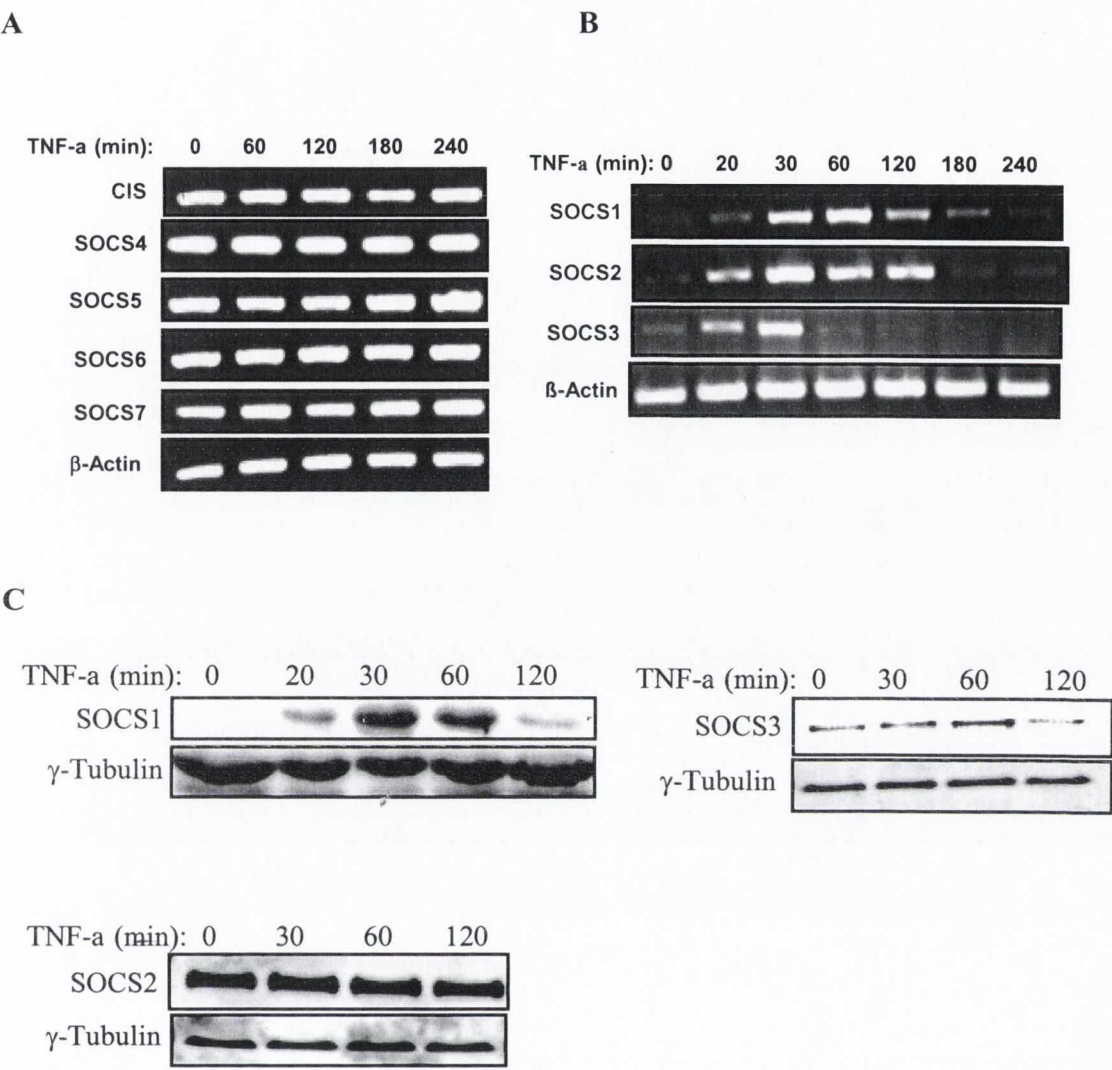
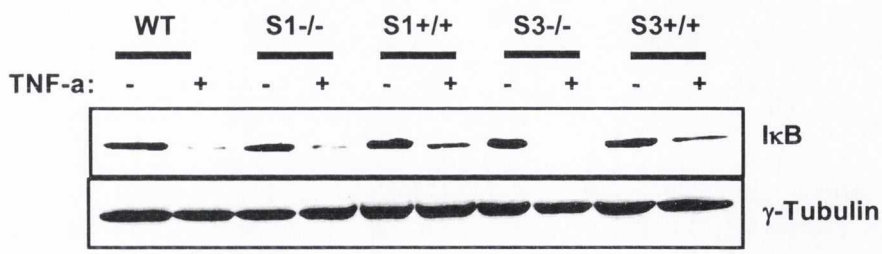


Figure P5.1: SOCS1 and SOCS3 mRNA and protein are regulated by TNF-α in PBMCs

(A) CIS, SOCS4-SOCS7, (B) SOCS1-SOCS3 mRNAs were analysed by RT-PCR from PBMCs treated with TNF-α (20ng/ml) over a time-course of 0 to 240min. (C) Protein lysates from PBMCs treated with TNF-α were analysed by immunoblotting for SOCS1, SOCS2, SOCS3 and γ-Tubulin using specific antibodies (n=3).

A



B

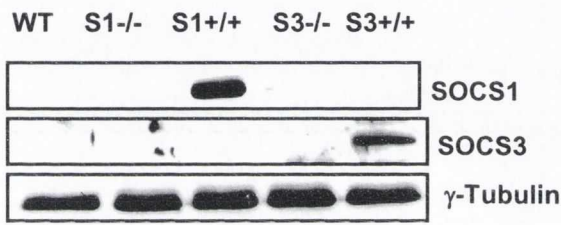


Figure P5.2: SOCS1 and SOCS3 regulate IκB degradation

(A) WT, SOCS1^{-/-}, SOCS1^{+/+}, SOCS3^{-/-}, SOCS3^{+/+} MEFs were treated +/- TNF-α (20ng/ml) for 20min before IκB and γ-Tubulin protein levels were quantified by immunoblotting. (B) SOCS1 and SOCS3 protein levels were verified by western blotting protein lysates using SOCS antibodies and γ-Tubulin antibody.

5.2 Hypothesis

SOCS1 and SOCS3 can regulate TNF- α -mediated signal transduction and we have observed elevated SOCS3 levels in HCV+ patient PBMCs and in Huh7 cells transfected with HCV. We propose that HCV-induced SOCS3 may down-regulate the TNF- α signalling pathway, resulting in reduced production of pro-inflammatory cytokines thus preventing inflammation induced pathology.

5.3 Aims

1. To determine whether TNF- α could induce SOCS3 expression similarly in healthy and HCV+ PBMCs
2. To measure pro-inflammatory cytokine induction in response to TNF- α in the PBMCs of healthy and HCV+ individuals
3. To measure pro-inflammatory cytokine induction in response to TNF- α in Huh7 cells expressing HCV proteins

5.4 Results

5.4.1 SOCS3 induction and TNF- α -mediated signalling is reduced in PBMCs from HCV+ patients

While HCV often results in chronic infection, patients regularly suffer mild symptoms, demonstrating the virus's ability to suppress immune pathology [244]. Having observed that SOCS3 inhibited TNF- α signal transduction, we wondered if it could suppress TNF- α 's pro-inflammatory effect of during HCV infection. Having previously found elevated SOCS3 levels in HCV+ PBMCs we investigated SOCS3 mRNA and protein regulation over a 0-3h time-course with added TNF- α (20ng/ml). SOCS3 mRNA was poorly induced by TNF- α in HCV+ PBMCs, compared to healthy controls (Figure 5.1A) and the high basal levels observed at the mRNA level were reflected in SOCS3 protein expression as there was no upregulation upon TNF- α treatment (Figure 5.1B), unlike the transient induction observed in healthy PBMCs (Figure P5.1C). These results suggest a novel mechanism used by HCV, to enhance SOCS3 expression and reduce further signal transduction of the pro-inflammatory cytokine TNF- α .

Having observed endogenously expressed SOCS3 in PBMCs of HCV+ patients, we wondered if SOCS3 was inhibiting the TNF- α /NF- κ B pathway in HCV infected patients. Therefore, we next analysed TNF- α -mediated I κ B degradation in PBMCs from healthy and HCV+ patients. We found that unlike I κ B degradation in PBMCs from healthy individuals, TNF- α treatment did not degrade I κ B in PBMCs from HCV+ patients (Figure 5.1C), indicating a block in the TNF- α /NF- κ B pathway. Taken together, these results strongly suggest that HCV-induced SOCS3 acts to block TNF- α signal transduction.

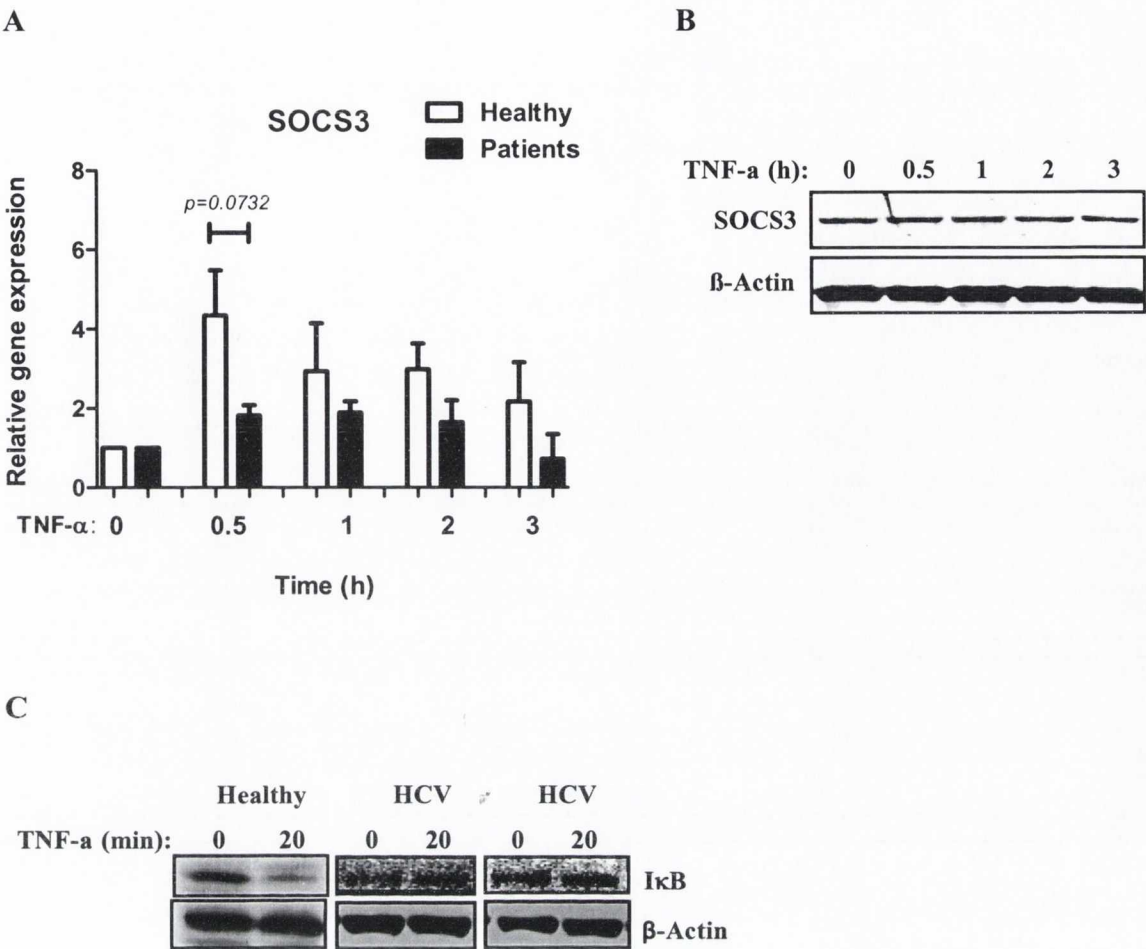


Figure 5.1: HCV+ PBMCs poorly induce SOCS3 and have reduced TNF-a induced I κ B degradation

(A) qRT-PCR analysis of SOCS3 mRNA expression in PBMCs from healthy (n=5) and HCV+ (n=7) following stimulation with TNF-a (20ng/ml) over a time course of 0.5, 1, 2 and 3h. Mean (SD) fold changes are shown for each stimulation, relative to the unstimulated sample (0). (B) SOCS3 protein levels were quantified in PBMCs from HCV+ patients treated over a time-course of 0-3h using the appropriate antibodies (n=3). (C) I κ B protein levels were measured by immunoblotting protein lysates of PBMCs from one healthy and two HCV+ patients, treated with TNF-a for 0 and 20min.

5.4.2 TNF- α pro-inflammatory cytokine induction is attenuated in HCV+ PBMCs

Since TNF- α signalling was reduced in the PBMCs of HCV+ patients, we investigated basal expression and TNF- α -mediated induction of pro-inflammatory cytokines. We treated PBMCs from HCV+ and healthy individuals with TNF- α for 0, 2 and 3h, before analysing mRNA of the proinflammatory cytokines and chemokines IL-6, TNF- α , CXCL8 and CXCL10. Basal levels of these cytokines and chemokines were similar in both groups (Figure 5.2). Each of these cytokine genes was transiently upregulated by TNF- α in PBMCs from healthy individuals. However, IL-6, CXCL8 and TNF- α tended to show a reduced induction in HCV+ PBMCs, however not significantly, while CXCL10 levels were similar in both groups (Figure 5.3). These findings indicate that the observed block in TNF- α -mediated signal transduction in HCV+ PBMCs, lessens the upregulation of pro-inflammatory cytokines.

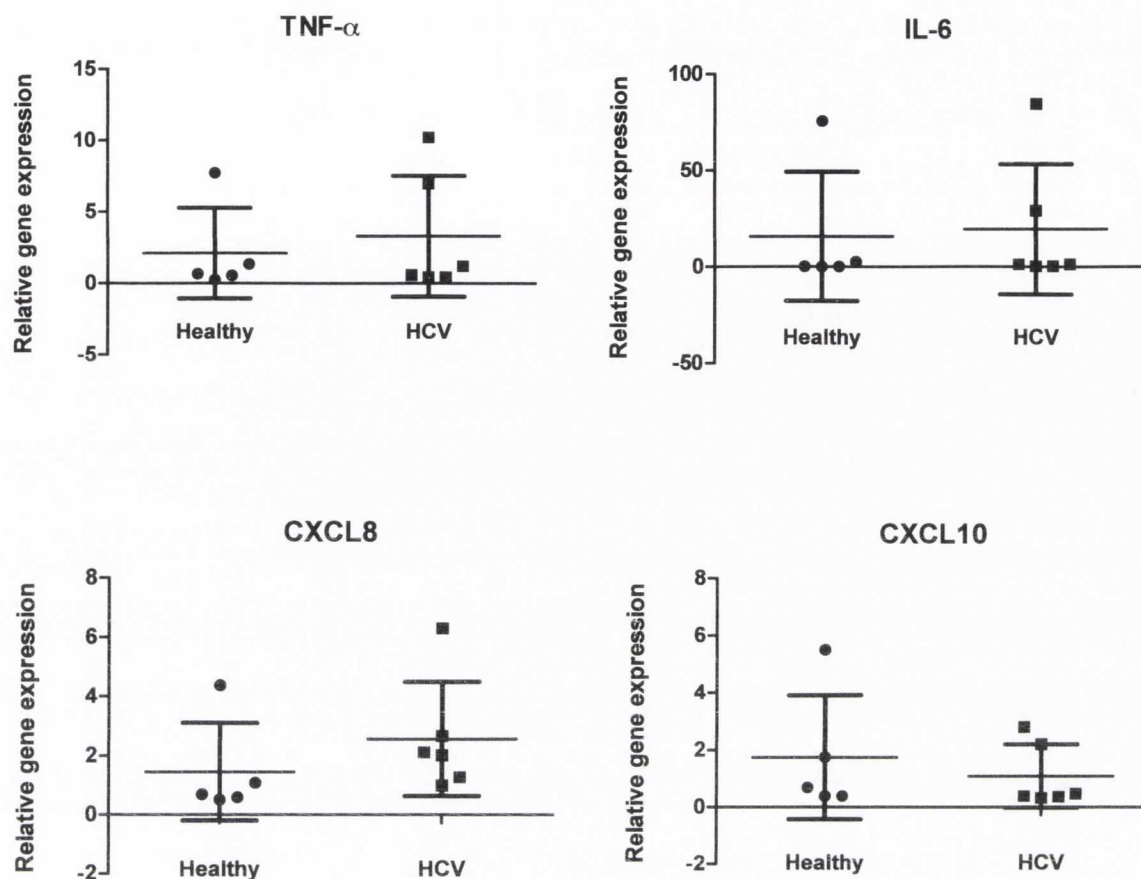


Figure 5.2: Basal cytokine mRNA expression is similar in PBMCs from healthy and HCV+ individuals

qRT-PCR analysis of TNF- α , IL-6, CXCL8 and CXCL10 basal mRNA expression in healthy (n=5) and HCV+ (n=6) PBMCs. Mean (SD) fold changes are shown for HCV+ samples relative to the average of the healthy controls.

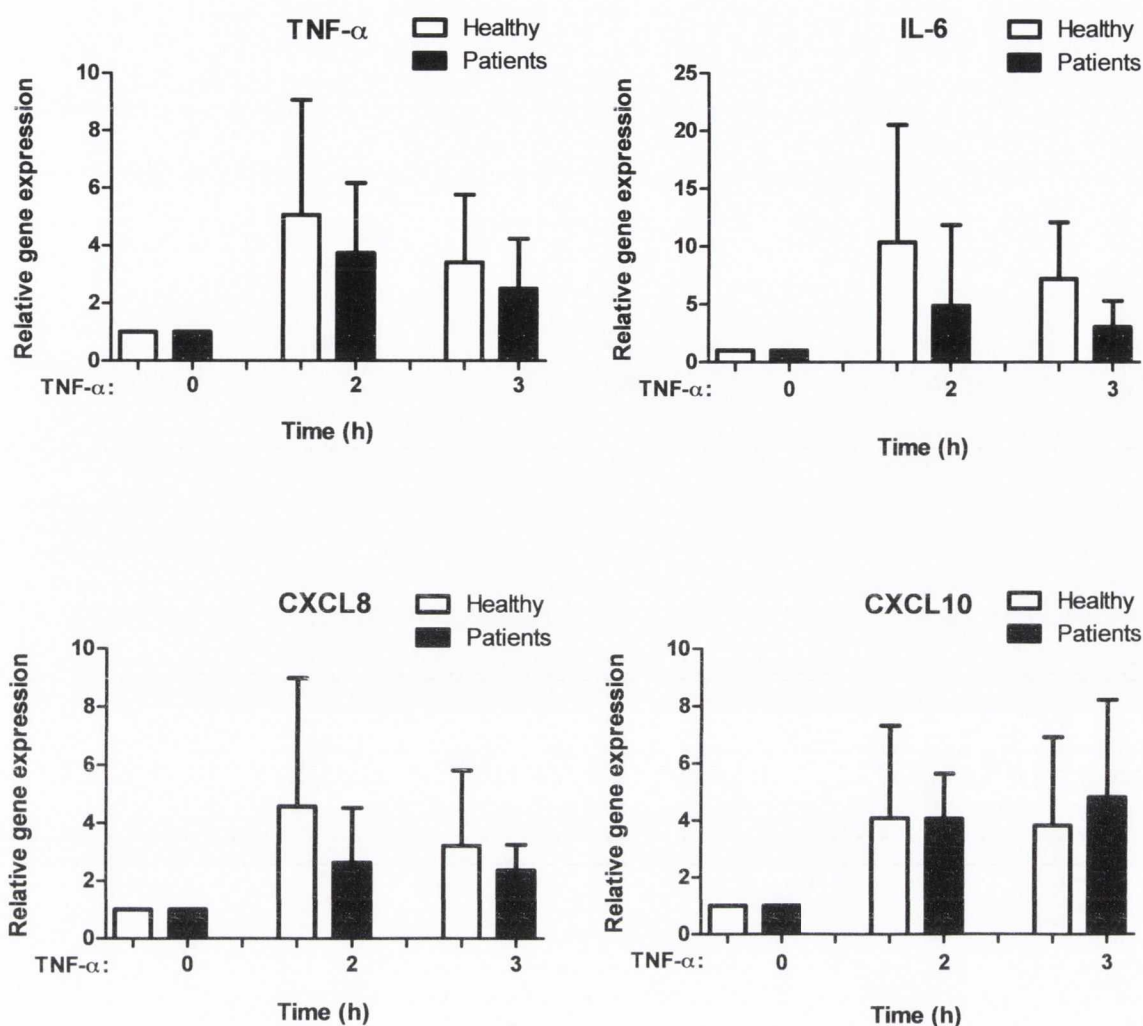


Figure 5.3: Induction of pro-inflammatory cytokines by TNF- α in healthy and HCV+ PBMCs

qRT-PCR analysis of TNF- α , IL-6, CXCL8 and CXCL10 mRNA induction in healthy (n=5) and HCV+ (n=7) PBMCs treated with 20ng/ml of TNF- α over a time course of 2 and 3h. Mean (SD) fold changes are shown for each stimulation relative to the unstimulated sample (0).

5.4.3 HCV expression induces SOCS3 and reduces CXCL8 production in Huh7 hepatocytes

HCV classically infects hepatocytes and we have previously observed elevated SOCS3 mRNA expression in Huh7 cells transfected with HCV (Figure 4.5(i)). We next examined HCV's effect on SOCS3 protein and TNF- α -induced cytokines in the hepatocyte cell line, Huh7. We transfected Huh7 cells with a pBRTM/HCV1-3011 DNA construct compared to EV control for 24h, and observed an increase in SOCS3 protein expression in HCV transfected cells, compared to EV controls (Figure 5.4A). Next, we measured CXCL8 and CXCL10 protein levels in the supernatant and found CXCL8 induction to be significantly reduced in Huh7 cells expressing HCV, while CXCL10 induction was unaffected (Figure 5.4B).

5.4.4 HCV-induced SOCS3 directly inhibits TNF- α -induced CXCL8 production

To investigate the direct role of SOCS3 in HCV-mediated immune subversion, we transfected Huh7 cells with SOCS3 shRNA and control shRNA for 24h. After confirming SOCS3 knock down by qRT-PCR and western blot (Figure 5.5A), we transfected Huh7s with HCV or EV for 10h, before stimulating with TNF- α (20ng/ μ l) for 18h. We analysed supernatants by ELISA and found that the reduction of CXCL8 in Huh7 cells expressing HCV was restored when SOCS3 was silenced using shRNA. In contrast CXCL10 levels were unaffected (Figure 5.5B). Collectively these results indicate that HCV may induce SOCS3 expression to negatively regulate pro-inflammatory responses, thereby increasing virus survival by prolonging disease progression.

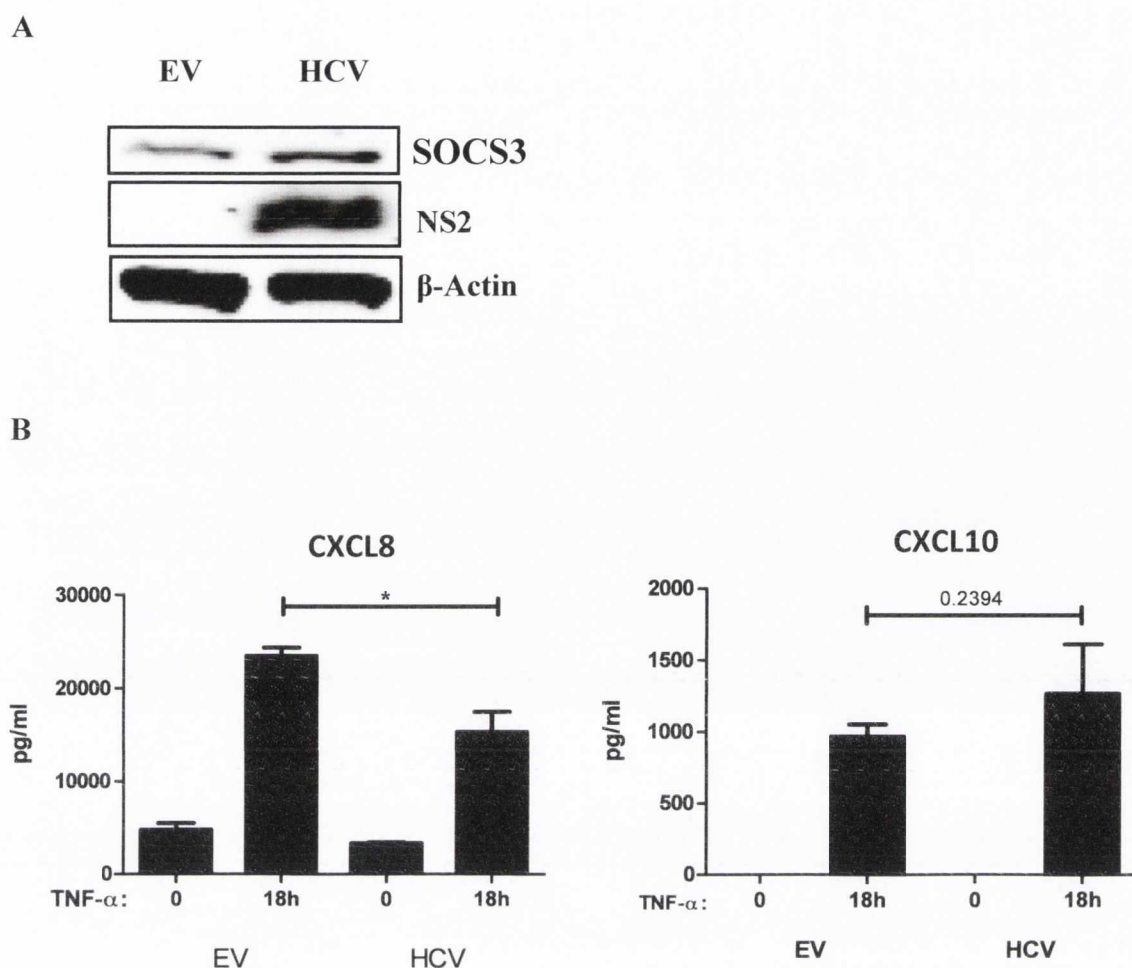


Figure 5.4: SOCS3 expression is increased while pro-inflammatory cytokine CXCL8 is decreased, in the presence of HCV.

(A) Huh7 cells were transfected with HCV and control EV for 24h followed by stimulation with TNF- α (20ng/ μ l) for 18h. SOCS3 protein lysates were measured at time 0 by immunoblotting. (B) CXCL8 and CXCL10 protein were analysed by ELISA following 18h stimulation with TNF- α . Experiments were performed in triplicate and graphs are representative of one independent experiment. *P* values were calculated using paired t-tests.

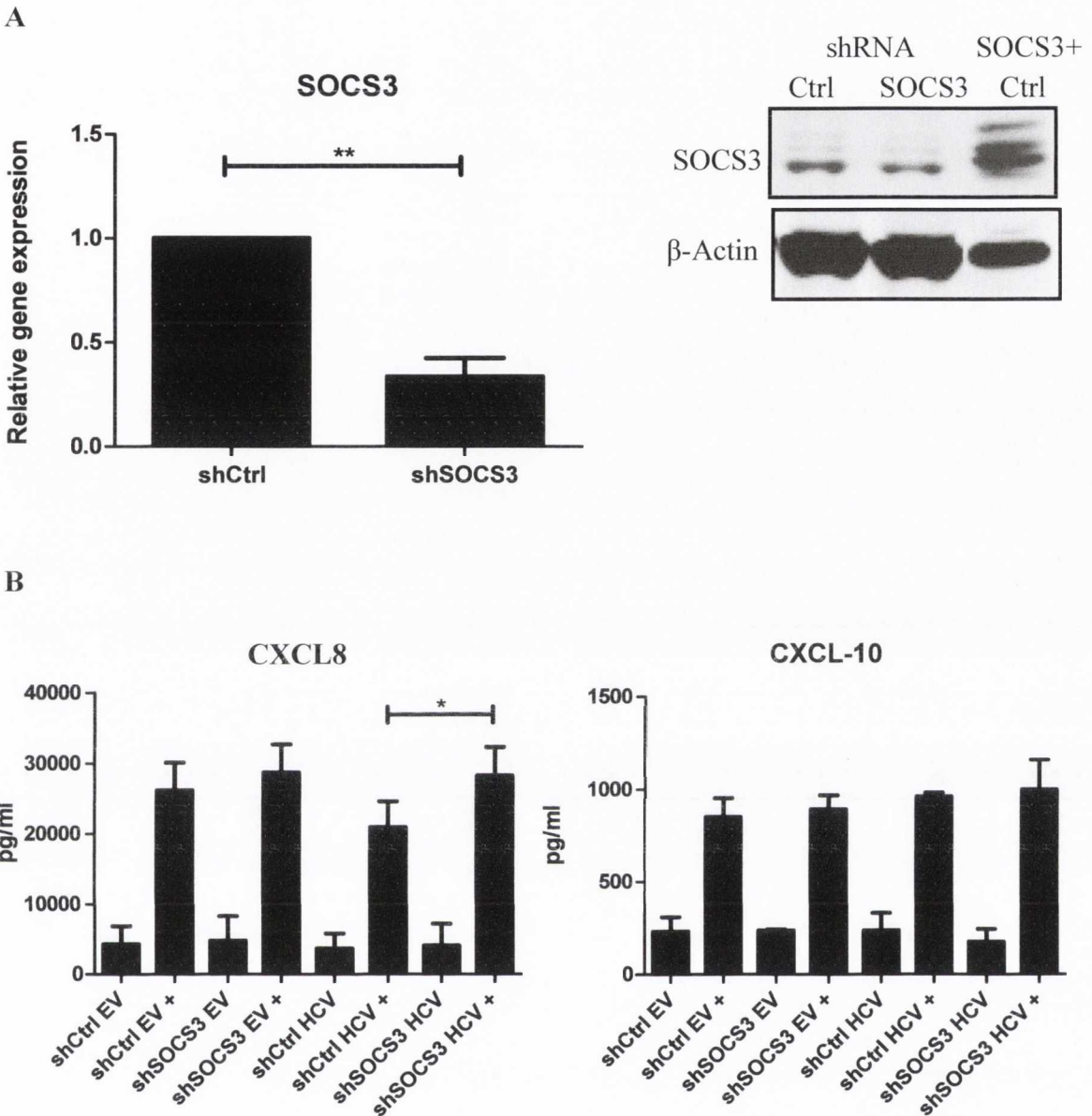


Figure 5.5: Induction of pro-inflammatory cytokine CXCL8 is restored when SOCS3 is silenced in HCV infected Huh7 cells

(A) Huh7 cells were transfected with SOCS3 shRNA (shSOCS3) and shRNA scrambled control (shCtrl) for 24h (n=3). SOCS3 mRNA and proteins were analysed by qRT-PCR and western blot, respectively. Relative gene expression was compared to shCtrl (Mean $\pm$ SD). (B) Huh7 cells were transfected with shSOCS3 and shRNA for 24h, then transfected with HCV and EV for 10h before being stimulated with TNF- α for 18h (+). CXCL8 (n=5) and CXCL10 (n=3) protein was quantified by ELISA. *P* values were calculated using paired t-tests.

5.5 Discussion

In this study, we demonstrate that SOCS1 and SOCS3 regulate TNF- α -mediated signal transduction. Elevated SOCS3 expression in PBMCs from HCV-infected patients reduced normal TNF- α -mediated I κ B degradation and pro-inflammatory cytokine production. Furthermore, increased SOCS3 expression in Huh7 cells transfected with HCV directly decreased pro-inflammatory CXCL8 induction. These findings implicate viral induction of SOCS3 in the blockade of TNF- α -mediated immunity and outline a novel mechanism of intracellular control that may regulate the inflammatory response during HCV infection, thus prolonging disease progression.

While TNF- α has a fundamental role during viral clearance, its regulation by SOCS in PBMCs has not been studied. Here we have identified for the first time SOCS1 and SOCS3 as inhibitors of TNF- α -induced CXCL8 in PBMCs, supporting previous findings of SOCS-mediated control of TNF- α regulated processes, including cell death in fibroblasts and sensitivity in pancreatic beta cells [243,245]. Indeed, SOCS1 deficient macrophages produce excessive amounts of IL-12 in response to TNF- α and TNF- α -induced SOCS3 generates resistance to IFN- α [246], further suggesting an important role for SOCS proteins in the control of both TNF- α signal transduction and subsequent inflammation [247]. SOCS3 has also been shown to negatively regulate the IL-1 signalling pathway via the TRAF6/TAK1 complex. SOCS3 inhibits the association of TRAF6 and TAK1 by preventing the ubiquitination of TRAF6 [228]. It is possible that SOCS3 negatively regulates the TNF- α /NF- κ B pathway by a similar mechanism.

HCV is known to block several immune cell functions, including cleavage of the NF- κ B signalling components, TRIF and IPS-1, and activation of the JAK-STAT pathway, however, the effect of HCV on TNF- α inflammatory responses is poorly understood [173,248-250]. Our study has revealed a mechanism by which HCV-induced SOCS3

blocks pro-inflammatory TNF- α signal transduction in PBMCs. Furthermore, the presence of HCV in Huh7 hepatocytes significantly increases SOCS3 levels and consequently reduces pro-inflammatory CXCL8 production. This effect was reversed when SOCS3 was silenced by shRNA, further supporting our hypothesis that HCV-induced SOCS3 negatively regulates the TNF- α signalling pathway to block pro-inflammatory immune responses.

Previous studies have shown that over-expression of NS5A and NS5B inhibits TNF- α signal transduction by blocking TRADD and TRAF2 and IKK-induced NF- κ B activation, respectively [251,252]. Regulation of pro-inflammatory signals through the induction of SOCS is an important homeostatic mechanism used to generate a controlled response to infection. Our findings indicate that HCV hijacks this process to create an environment that permits chronic viral infection with minimal pathology. Indeed, a prominent characteristic of HCV infection is the prolonged nature of disease progression, often without any obvious signs of infection [253]. Our results indicate that this silent progression to chronicity may be mediated through HCV's ability to suppress the inflammatory response to TNF- α , via SOCS3 induction.

In conclusion, these findings reveal a novel mechanism by which SOCS regulates TNF- α and controls its pro-inflammatory signal transduction. This system is manipulated by HCV-induced SOCS3 which suppresses TNF- α signalling. We have discovered a novel regulatory mechanism used by HCV to suppress normal immunity, perhaps explaining why HCV infection is associated with mild pathology and can remain undetected for many years.

Chapter 6

General Discussion

Chapter 6: General Discussion

Inflammation is characterised by a cascade of events including cellular activities and microvascular reactions, which occur in response to injury or infection and serve to remove the invading pathogen and repair damaged tissue. Unresolved inflammation results in organ dysfunction and cell death; hence there are a number of endogenous mechanisms in place to regulate the inflammatory response. SOCS proteins are induced by a wide range of stimulants including cytokines, growth factors and interleukins, and act to attenuate signal transduction and inflammation, thereby ensuring an appropriate immune response. The SOCS family consists of eight members (CIS, SOCS1-7); however, SOCS1 and SOCS3 are best characterised owing to their roles as negative regulators of the JAK-STAT pathway. The JAKs and STATs are essential intracellular mediators of inflammatory pathways and the antiviral immune response. SOCS1 is known to directly interact with JAK proteins and inhibit their kinase ability whereas SOCS3 has a greater binding affinity for the receptor subunit, and prevents STATs from docking on the receptor [112]. This is achieved via a specific KIR domain located next to the extended SH2 domain structure of SOCS1 and SOCS3 only.

SOCS proteins have also been shown to regulate the TLR and NF- κ B signalling pathways, highlighting their role in controlling the inflammatory response. SOCS proteins regulate pathways in a classic negative feedback loop, i.e. their expression is induced by the pathway they are destined to inhibit. Research has focused on the role of SOCS proteins as negative regulators of the immune response, however, little is known about their own regulation. Dysregulation of SOCS proteins has been noted in inflammatory diseases such as colitis, RA and Crohn's disease, emphasising their

functional importance in a normal immune response and their potential as therapeutic targets in chronic inflammatory conditions [115].

miRNAs are a class of regulatory molecules that have recently emerged as key post-transcriptional regulators of $\geq 30\%$ of gene expression. miRNAs have been documented in several biological processes such as cell proliferation, development and apoptosis as well as facilitating the cell fate of immune cells. Specific miRNAs, miR-146a, miR-21 and miR-155, are induced in response to LPS and contribute to the control of the TLR signalling pathway [138,147]. miR-19a has been reported to be induced by IFN- α and has been shown to regulate SOCS1 expression, therefore, we wondered if miR-19a could regulate additional proteins associated with the JAK-STAT pathway [146]. Using bioinformatic software, KEGG database, InnateDB and TargetScan, our research identified twelve genes that code for components of the JAK-STAT pathway to contain miR-19a target sequences in their 3'UTRs. Further investigation into four classical regulators of the JAK-STAT pathway, SOCS1, SOCS3, SOCS5 and Cul5, using a miRNA mimic, revealed that miR-19a significantly down regulates SOCS3 mRNA and protein expression.

We also discovered that when SOCS3 expression is silenced by miR-19a, pSTAT3 levels are increased in response to IFN- α and IL-6 in Huh7 cells. These results were consistent over 24, 48 and 72hours. The purpose of miRNAs may be to allow a strong initial immune response which is gradually dampened down, or to “fine tune” and assist established mechanisms of regulation, such as SOCS proteins. Indeed SOCS3 has been shown to be down-regulated by miR-203 in psoriatic keratinocytes, and this has been speculated to concur with up-regulation of STAT3, also detected in psoriatic lesions; however this remains to be proven [136]. We have revealed a novel mechanism of control of a classic JAK-STAT pathway regulator, SOCS3, with subsequent enhanced

expression of a key transcription activator, STAT3, by miR-19a. Interestingly, no miR-19a targets were identified in components of the TLR pathway, indicating that miR-19a may specifically regulate JAK-STAT signalling. miR-19a is transcribed from the oncogenic miR-17-92 cluster, which is shown to be expressed in lung cancer and B cell lymphomas [143]. Furthermore, miR-19a itself is reported to be expressed in MM patients and postulated to contribute to SOCS1 down-regulation and IL-6 activation at later stages in MM pathogenesis [146]. Our findings demonstrate that miR-19a down-regulates SOCS3, leading to increased STAT3 expression, which may have implications in inflammatory diseases and malignancies associated with aberrant JAK-STAT expression. Future work in this area would be to investigate miR-19a levels in patients suffering from diseases associated with inactivation of SOCS3 regulation, such as RA, viral hepatitis and cancer, with the potential of generating therapeutic intervention aimed at reducing miR-19a levels in these patients [254].

SOCS proteins are not only targeted and regulated by miRNAs; SOCS1 and SOCS3 are also targets of HCV, which hijacks their ability to negatively regulate an essential antiviral pathway. The cytokine IFN- α signals via the JAK-STAT pathway inducing the production of essential ISGs including PKR, MxA and OAS, to achieve an antiviral state in the cell. In light of the powerful role of SOCS1 and SOCS3 in inhibiting the JAK-STAT pathway, viruses have exploited their function to overcome the immune response. Indeed, SOCS1 and SOCS3 have been implicated in the immune response to IFN- α during HCV infection. Elevated levels of SOCS3 in the liver are associated with a poor response to IFN- α therapy due to impairment of IFN responsiveness [218]. Moreover, HCV's core protein is documented to induce SOCS3 and suppress JAK-STAT signalling [168,218]. However, the expression of SOCS1 and SOCS3 in immune cells is poorly explored and we set out to investigate endogenous levels of these

proteins, and their induction in response to IFN- α , in PBMCs from healthy and HCV infected individuals. We found that PBMCs from HCV+ individuals express significantly higher basal mRNA levels of SOCS3 when compared to healthy controls. SOCS1 levels however, were similar in both groups indicating that HCV acts to induce SOCS3 specifically. Induction of SOCS1 and SOCS3 mRNA, following IFN- α stimulation, was similar in healthy and HCV+ PBMCs suggesting that this part of the JAK-STAT signalling pathway is intact. Furthermore, we examined the expression of SOCS1 and SOCS3 in the hepatocyte cell line Huh7, following transfection with a construct containing HCV proteins, and found SOCS3 basal mRNA and protein levels to be significantly elevated, when compared to cells transfected with EV.

To investigate the mechanism behind specific SOCS3, but not SOCS1, up-regulation during HCV infection, we used a bioinformatic tool to search for transcription factor binding sites in SOCS1 and SOCS3. Preliminary results revealed that indeed SOCS1 contains potential binding sites for transcription factors, IRF1 and IRF2, whereas SOCS3 primarily contains binding sites for STAT proteins. This may reveal a mechanism by which HCV achieves selective up-regulation of SOCS3, however, further experimental investigation is required.

Following IFN- α stimulation, SOCS1 and SOCS3 were induced similarly in HCV and EV transfected Huh7 cells. Bode *et al.*, reported that HCV core protein induced elevated levels of SOCS3 expression in HepG2 cells, consistent with the inhibition of IFN- α -induced STAT1 tyrosine phosphorylation [177]. Additionally Vlotides *et al.*, reported a decrease in STAT1 and STAT3 phosphorylation in SOCS1 and SOCS3 overexpressing HepG2 cells, with subsequent down-regulation of antiviral ISGs, MxA and OAS, and postulated this to be the mechanism that confers IFN- α resistance during antiviral treatment for HCV [180]. An additional mechanism for the up-regulation of

SOCS3 during HCV infection has been suggested by Funaoka *et al.*, who generated HCV core mutants and examined their effects on JAK-STAT signalling in the presence of HCV. The expression of IL-6 and SOCS3 were significantly increased and thus IL-6-induced SOCS3-mediated suppression of IFN was postulated as a mechanism for IFN resistance [220]. IL-6 is secreted in response to cellular steatosis and insulin resistance, both of which are observed in HCV patients, however, this remains to be investigated [179].

SOCS3 was induced in response to IFN- α , in both HCV+ PBMCs and Huh7 cells expressing HCV, indicating that further up-regulation of SOCS3 through the JAK-STAT pathway is not inhibited in these cells, despite elevated endogenous levels. Therefore, we wondered of what benefit SOCS3 could be to HCV. While it is evident that HCV is acting to induce SOCS3 specifically, this does not appear to hamper signalling via the JAK-STAT pathway, hence, we questioned what other targets of SOCS3 might be affected by these enhanced levels.

SOCS proteins have been documented to regulate the TNF- α /NF- κ B pathway and we postulated that high SOCS3 basal levels may have an effect on TNF- α signal transduction. TNF- α is a pleiotropic cytokine and plays a pivotal role in the inflammatory response. TNF- α is responsible for a multitude of events during inflammation including recruitment of neutrophils, expression of adhesion molecules, activation of macrophages and chemotaxis. TNF- α is also associated with a number of inflammatory disease such as RA, IBD, SLE and MS and has therefore become a target for therapeutic intervention in these conditions. Indeed, anti-TNF- α drugs are successful and widely administered for the treatment of RA.

SOCS1 has been shown to suppress NF- κ B activity by binding to the p65 subunit and modulating its ubiquitination and degradation, while SOCS3 inhibited TNF- α -induced phosphorylation of the MAPKs, ERK1, ERK2, p38 and JNK in beta cells and in primary rat islets [117,118]. Initially we investigated the induction of SOCS in immune cells, in response to TNF- α , and found SOCS1 and SOCS3 mRNA and protein to be transiently up-regulated. Moreover, in cells overexpressing SOCS1 and SOCS3, I κ B degradation was inhibited, indicating that SOCS1 and SOCS3 are induced by and have a negative impact on, the TNF- α /NF- κ B signalling cascade. We then examined the induction of SOCS3 in response to TNF- α in PBMCs from healthy and HCV+ individuals. Our results revealed that SOCS3 had a tendency towards lower induction in HCV+ immune cells, when compared to healthy controls. Furthermore, I κ B degradation was reduced in the PBMCs from HCV+ individuals.

To determine what effect this was having downstream of the pathway, we subsequently measured the pro-inflammatory cytokines and chemokines IL-6, CXCL8, CXCL10 and TNF- α , and found basal mRNA levels to be similar in both healthy and HCV+ PBMCs. However, upon stimulation with TNF- α , induction of IL-6, CXCL8 and TNF- α was dampened in HCV+ PBMCs when compared to controls, indicating that HCV may upregulate SOCS3 expression to block responsiveness to TNF- α , providing an appropriate environment for the virus to survive and replicate. A prominent characteristic of HCV infection is the prolonged nature of disease progression, often without any obvious signs of infection, implying that HCV has developed mechanisms to evade immune detection while inflicting minimal pathology. The advancement of HCV infection, calculated using a Markov model, indicated that the risk of developing cirrhosis over a 30-year period was 13–46% for men and only 1–29% for women, with

a low lifetime prediction of end-stage liver disease complication and of liver-related deaths [187].

As HCV primarily infects the liver, and we have shown elevated levels of SOCS3 in Huh7 cells expressing HCV, we examined pro-inflammatory cytokine induction in response to TNF- α in this model. In Huh7 cells expressing HCV proteins we found a significant decrease in CXCL8 induction, in response to TNF- α . CXCL10 levels were similar in both groups. Interestingly, CXCL10 can also be induced by TNF- α via the MAPK pathway, which may explain why CXCL10 levels were unchanged [255]. To correlate the decrease in CXCL8 induction with elevated SOCS3 levels, we used shRNA to silence SOCS3 expression. This restored CXCL8 signalling, confirming a direct link between HCV-induced SOCS3 and reduced pro-inflammatory production. Previous studies have not analysed the effects of SOCS proteins on TNF- α signalling in PBMCs, revealing our findings to be novel. HCV can remain undetected in the body for many years, indicating a successful suppression of the immune system. Our research suggest that HCV up-regulates SOCS3 in a bid to inhibit pro-inflammatory cytokine signalling, which leads to a robust inflammatory immune response under normal circumstances. This may explain the mechanism behind the mild inflammation and slow disease progression often associated with HCV infection.

Based on these interesting results, future work on this project would involve determining the mechanism by which SOCS3 inhibits the TNF- α /NF- κ B pathway. It has been shown that SOCS3 can negatively attenuate the IL-1 signalling pathway, by binding to TRAF6 and preventing its associated with TAK1, thus inhibiting downstream signalling [228]. It is likely that a similar mechanism is used in HCV+ PBMCs. Furthermore, SOCS3 expression levels should be correlated with liver fibrosis scores and ALT levels in patients who have clinical symptoms of mild inflammation

and those who have chronic inflammation, to establish whether high SOCS3 levels may predict inflammation and thus progressive liver damage. Additionally, it would be interesting to investigate the inflammatory diseases RA and IBD, which are associated with aberrant TNF- α expression, in HCV infected patients to elucidate the possibility of developing a therapy based on SOCS3 regulating TNF- α signalling.

In conclusion, this project has shown SOCS3 to be controlled by miR-19a, TNF- α and HCV. We discovered miR-19a to be a novel regulator of SOCS3, which may contribute to the knowledge and understanding of the regulation of this powerful inhibitor of the JAK-STAT pathway, and help to elucidate the mechanism behind SOCS3 deregulation. We also observed significantly elevated SOCS3 expression levels in PBMCs from HCV+ individuals, and in Huh7 cells transfected with HCV, confirming SOCS3 to be targeted by HCV during viral infection. HCV-induced SOCS3 may contribute to the mild pathology associated with chronic HCV infection by suppressing the TNF- α /NF- κ B pathway and thus preventing pro-inflammatory cytokine production; elucidating a novel regulatory mechanism used by HCV to suppress immunity and promote disease progression. Collectively these results highlight the importance of SOCS3 in regulating the inflammatory immune response; however, this inhibitory molecule is also an attractive target for HCV and has been exploited for its powerful suppressive role in antiviral immunity. Collectively our findings might pave the way for the discovery of novel HCV-derived anti-inflammatory molecules.

Chapter 7

References

Chapter 7: References

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