A novel role of SOCS-4 and SOCS-5 in Cytokine Signalling

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Declaration

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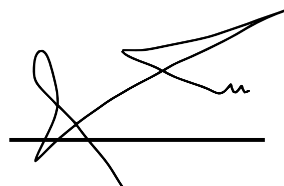

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Abstract

The immune system is propagated by cytokines and their intracellular signal transduction. To ensure an appropriate immune response, these signalling pathways are tightly controlled. One such regulatory mechanism is the induction of a family of 8 intracellular proteins called Suppressor of Cytokine Signalling (CIS [Cytokine-inducible SH2 protein] and SOCS1-7). Many cytokines and pathogenic stimuli have been shown to regulate SOCS expression, which subsequently act by binding to components of the intracellular pathway and thus block the cascade of signalling events. CIS, SOCS1-3 are the most comprehensively described SOCS proteins, with SOCS4-7 being less well understood. Therefore, this project aimed to investigate the role of 2 of these "neglected SOCS", SOCS4 and SOCS5 and their involvement in the signal transduction of pro-inflammatory (TNF-α, IL-6 and IL-1β) and anti-viral (IFN-α) cytokines. Indeed, we discovered that both SOCS4 and SOCS5 mRNA and protein expression were induced by these cytokines, to varying degrees. Interestingly, while SOCS4 overexpression inhibited IL-6-mediated STAT3 phosphorylation, SOCS5 expression enhanced IL-6-mediated pSTAT3 levels. However, SOCS5 expression acted to reduce TNF-α-mediated IκBα degradation. Together these findings reveal for the first time, that both SOCS4 and SOCS5 are induced by pro-inflammatory and anti-viral cytokines and that SOCS5 can regulate pro-inflammatory signalling of both IL-6 and TNF-α, and while SOCS4 has no inhibitory effect upon TNF-α signaling, it acts to reduce pro-inflammatory responses of IL-6. These discoveries have not only revealed that SOCS4 and SOCS5 expression are regulated,

but have shed light upon new immune regulatory mechanisms for both of these proteins.

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Chapter 1
Introduction

1.1- The immune response to infection.

The immune system uses cellular and molecular processes to destroy pathogens and thus provide natural resistance against infection [1]. The immune system has two main arms: the innate and acquired (adaptive) immune responses [2]. The innate immune response has systemic effects on the entire body, which infiltrate organs, cells, proteins and tissues and can be described as a factory of defence [3]. Once the pathogen invades the epithelial barrier, a cascade of events is triggered, activating the innate immune response. Macrophages and neutrophils are the first on the scene and immediately combat the invasion by phagocytosis of the pathogen [3] . In addition, all innate immune cells have a unique set of conserved receptors, named pathogen recognition receptors (PRR), that recognise conserved components of the pathogen, called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), bacterial DNA and viral RNA. The most studied PRRs are Toll-Like Receptors (TLRs), which bind to microbial ligands, such as LPS, and activate many processes, including pro-inflammatory cytokine production, often leading to inflammation. Moreover, the binding of pathogenic ligands to TLRs can trigger the activation of dendritic cells (DCs), leading to the induction of adaptive immune responses [4]. TLRs are characterised by an extracellular luminal ligand-binding domain that contains leucine-rich repeat (LRR) motifs and an intracellular Toll/interleukin-1(IL-1R) receptor homology (TIR) domain [5]. TLRs are subdivided according to their location, some are situated at the cell surface and are activated in response to microbial protein lipids, including TLR4. While TLR3, 7, 8 and 9 are

located in the membrane of the endosome and they detect microbial nucleic acids such as synthetic double-stranded RNA, viral related single-stranded RNA and bacterial unmethylated CpG-DNA. Activation of these endosomal TLRs leads to the production of type I IFNs and pro-inflammatory cytokines (**Fig.1.1)**

Recognition of PAMPs by TLRs leads to the transcriptional upregulation of distinct genes, dependent on the downstream signalling cascades. The diversity in signalling cascades can be partly explained by the TIR domain containing adaptor proteins that are recruited to the cytosolic portion of the TLR. All TLRs, except TLR3, use the adaptor myeloid differentiation primary response protein 88 (MyD88) and therefore signal via the MyD88-dependent pathway. Interestingly TLR4 has been shown to activate both the MyD88-dependent and -independent pathways **(Fig.1.1)**. However, TLRs at the cell surface or at the luminal surface of endo-lysosomal membrane do not detect intracellular cytosolic pathogens, indeed these are detected by a group of cytosolic PRRs [6]. Cytosolic PRRs are divided into RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). The RLR family consist of three members that sense viral RNA, whereas the NLR family detect numerous PAMPs, non-PAMP particles and cellular stress [7, 8] **(Fig.1.1)**. Another example of cytosolic PRRs is the DNA-sensing system, which can activate innate immune responses by triggering the induction of Type I IFNs, for example being the DNA-dependent activator of IFN-regulatory factors (DAI) [9].

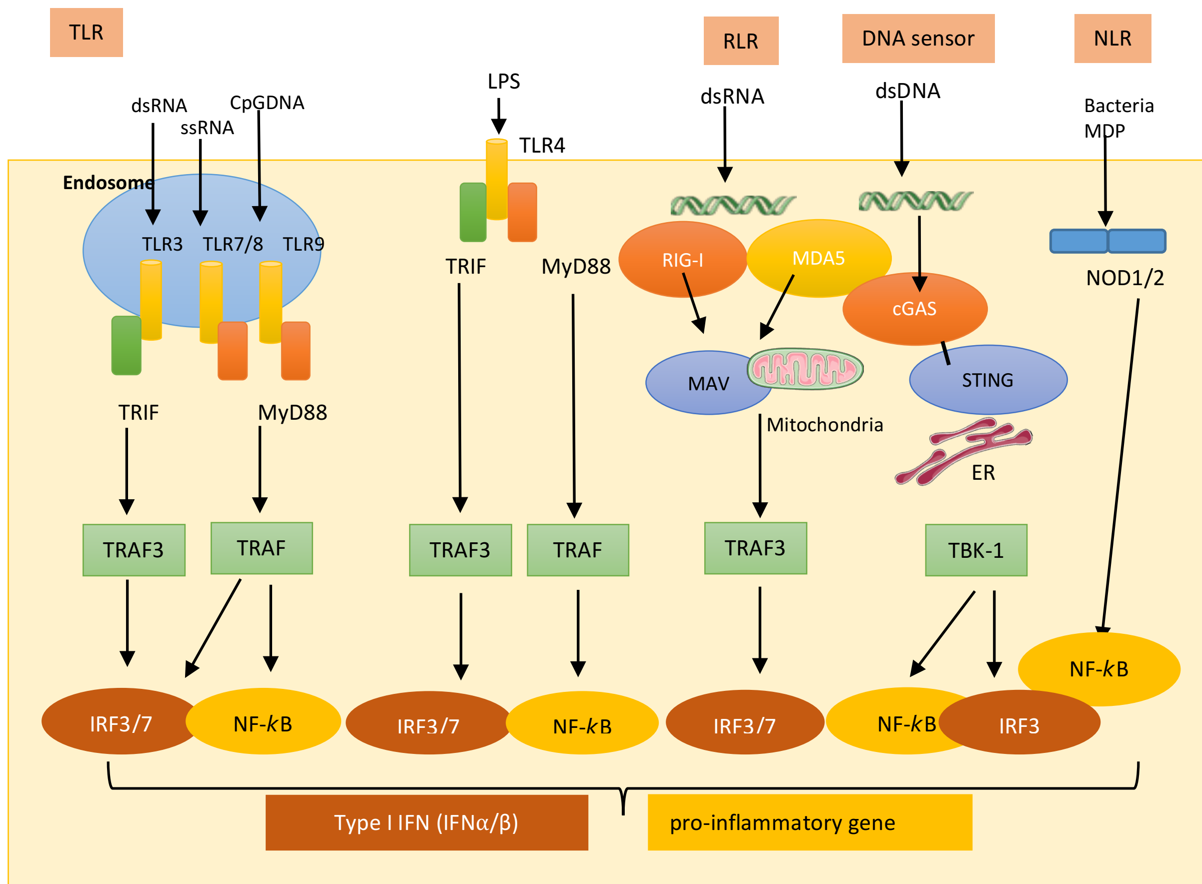

Figure 1.1- Pattern-recognition receptor (PRR) signaling pathways.

Three classes of PRR families, TLRs, RLRs and NLRs, sense microbial products or endogenous danger signals to trigger downstream signalling pathways. TLR3,7,8 and 9 are endosomal and detect microbial nucleic acid, while TLR4 is located on the cell surface and detects components of the bacterial cell wall. Upon stimulation, TLRs activate two pathways via MYD88 and/or TIR domain-containing adaptor protein inducing IFN-β (TRIF) resulting in the production of Type I IFNs and pro-inflammatory cytokines. RIG-I is a cytoplasmic viral RNA sensor that detects 5' triphosphate dsRNA. Following the binding of RNA, RIG-I is dephosphorylated and becomes active leading to its interaction with the adaptor mitochondrial anti-viral signalling protein (MAVS). MAVS activates TRAF3, which activates downstream IRF3 and induces Type I IFNs. NLR is activated by a small fragment of the bacterial cell wall, muramyl dipeptide (MDP). Upon activation, NOD1 and 2 homo-oligomerise and drive downstream NF-κB-dependent expression of pro-inflammatory cytokines. DNA-sensors recognize intracellular DNA and can lead to the activation of the protein cGAS, which then binds to the endoplasmic reticulum (ER)-localised adaptor protein, STING. The

cGAS-STING activation triggers phosphorylation and activation of TBK-1 and downstream IRF3 and NF-κB.

1.1.1- Innate signal transduction

PRRs activate several distinct intracellular signalling pathways, often named after their transcription factor components. Examples of three major transcription factors are: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPK), which play essential roles in induction of the pro-inflammatory response. While, IFN regulatory factors (IRFs) stimulate the production of IFNs [6].

TLRs signal through major adaptor proteins, either dependent or independent of MyD88 or TIR domain-containing adaptor inducing IFN-β (TRIF) [10]. Cytosolic PRRs, including RLRs and NLRs, trigger signalling through interaction between RIG-1/Melanoma Differentiation-Associated protein 5 (MDA5) and adaptor protein IFN-β Promoter Stimulator (IPS)-1 [11]. Another adaptor protein has been identified, called stimulator of interferon genes (STING), resides in the endoplasmic reticulum and can activate both NF-κB and IRF3 transcription pathways, exerting an effective anti-viral state via the induction of Type I IFN (IFN-α and IFN-β) [12].

Transcription factors play vital roles in regulation of gene expression, inducing the production of pro-inflammatory mediators, such as cytokines and IFNs and thus enhancing the immune response in order to achieve clearance of the pathogen.

1.1.2-The bridge between innate and adaptive immune responses

The link between innate and adaptive immune response can be achieved by PRR-mediated maturation of DCs and subsequent activation of pathogen-specific T lymphocytes (T cells) [13]. DCs are activated by the uptake of antigen, then migrate to lymph nodes in order to present antigenic peptides on MHC molecules to T cells. Priming of naïve T cells requires an interaction of co-stimulatory molecule (e.g. CD40 and CD86) on antigen-presenting cells (APCs) and cytokines (such as interleukin (IL-12) which are produced by activated DCs. By presenting the pathogenic antigens DCs stimulate naïve CD4+ T lymphocytes to differentiate into T helper (Th) subsets. Cytokines also play a critical role in activating different Th subset, IL-12 is responsible for inducing Th1 responses and production of IFN-γ while IL-23 and IL-1 contribute in inducing Th17 differentiation. In contrast, the Th2 response mostly relies on IL-4 [14, 15]. Indeed, the role of cytokines in orchestrating the maturation of the innate immune response and stimulating the differentiation of lymphocytes highlights their importance in controlling an appropriate response to infection.

1.2- Cytokines:

Cytokines are small molecules that regulate the expression and progression of the immune responses against pathogens. Cytokines contribute to many physiological processes involving the regulation of the immune system and inflammatory response. Indeed, their importance is evident in their detection during diagnostic screening for diseases and their use as therapeutics [16]. Cytokines can have autocrine effects (affecting the cell they are secreted by) or paracrine effects (affecting surrounding cells) and endocrine effects (affecting

distant cells within the body). The cytokine network is complex, often with different cytokines performing the same or similar functions. Others are pleiotropic, which describes how one cytokine can act on different cells thus generating different effects. Cytokines can also act in synergy to enhance a response and can even antagonise each other [17].

Broadly, cytokines can be pro-inflammatory (e.g. Interleukin 12, tumor necrosis factor (TNF-α) and IFN-γ) or anti-inflammatory (e.g. IL-10 and transforming growth factor (TGF-β)), whereas others can act as growth factors (e.g. Granulocye-Colony Stimulating Factor (G-CSF)) [16]. Cytokines can stimulate their biological effects through specific receptors, expressed by many cell types. Cytokine receptors can be characterised by one extracellular domain, a membrane spanning domain, and a cytoplasmic tail. 5 families of cytokine receptors have been identified **(Fig.1.2)**, including immunoglobulin (Ig) superfamily receptors-interleukin-1, Type I/II cytokine receptor family, TNF receptor family, receptor tyrosine kinase family and TGF-β receptor family [18]. Several pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, are produced by activated macrophages [17].

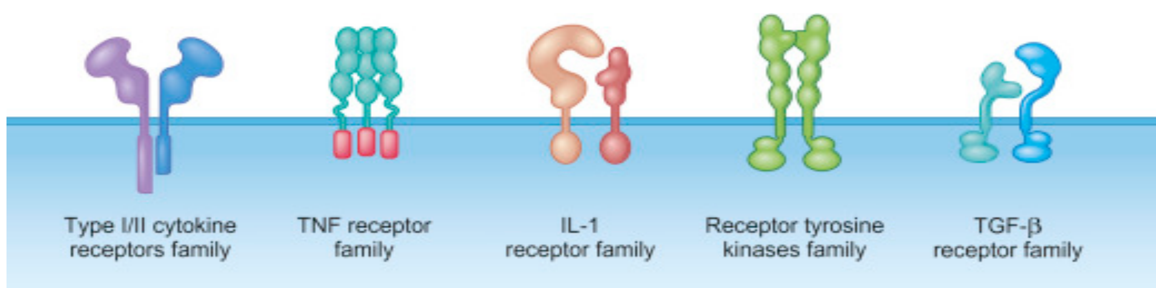

Figure 1.2- Five different classes of cytokine receptors. There are five families of receptors proteins that bind specifically to cytokines and transduce their signals. 1) Type I/II cytokine receptor family, 2) TNF receptor family, 3) IL-1 receptor family, 4) receptor tyrosine kinase family and 5) TGF-β receptor family **[19].**

1.2.1- Cytokines that mediate and regulate innate immune responses

1.2.2- TNF-α:

TNF-α is arguably one of the most important pro-inflammatory cytokines; being a key factor in the regulation of inflammation and often linked to autoimmune disorders, such as Rheumatoid Arthritis [20] and Inflammatory bowel disease [21]. TNF-α is produced by a vast array of cells, including macrophages, mast cells, T and B lymphocytes and natural killer (NK) cells, and its level in serum is elevated in inflammatory and infectious states [22]. TNF-α acts via a receptor that is made up of two transmembrane receptors (TNFR1 and TNFR2). TNFR1 is endogenously expressed in almost all mammalian tissue, whereas, TNFR2 expression is more commonly expressed in the cells of the immune system [23]. Binding of TNF-α to TNFR1 results in the release of a protein designated Silencer of Death Domain (SODD) [24]. This allows different cytoplasmic proteins to bind, such as TNFR-associated DD protein (TRADD), that propagates the signalling by recruiting two further proteins, the serine/threonine kinase receptor interacting protein-1 (RIP1) and the E3 ubiquitin ligase, TRAF2, that results in a complex of TRADD–RIP-1–TRAF2 released from TNFR1 [25]. Subsequently, a series of events occur involving recruitment of Mitogen-activated protein kinase kinase kinase3 (MEKK-3) and TGF-β activated kinase (TAK)1, and activation of the β-subunit of the inhibitor of κB (IκB) kinase (IKK) complex. This leads to phosphorylation and ubiquitin-mediated

degradation of the IκB proteins, which allows the translocation of the transcription factor NF-κB to the nucleus. NF-κB then binds to promoter regions in DNA, which initiates gene transcription **(Fig. 1.3)** [26, 27]. In addition, the TRADD–TRAF2 complex can activate MAP2Ks, including MEK-3 and -6, by recruiting apoptosis-signaling kinase-1 (ASK-1) [28]. The phosphorylation of MEKs leads to activation c-Jun N-terminal kinases (JNKs) and p38 MAPKs, thus activating the transcription factor activating protein 1 (AP-1), that facilitates gene transcription **(Fig. 1.3)** [29]. Both transcription factors, AP-1 and NF-κB, mediate cell survival and pro-inflammatory signalling. TNFR2, lacks an intracellular death domain and signals via direct interaction with the TNFR-Associated factor (TRAF) family [30]. TRAF2 can limit MAP3K signalling via recruitment and association with cellular inhibitors of apoptosis protein 1 (cIAP1), which promotes the degradation of TRAF2 and ASK1 by mediating ubiquitin ligase (E3) activity [31].

Interestingly, TNFR1 has also been shown to induce the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, through binding of the N-terminal cytoplasmic domain of TNFR1 to JAK leading to tyrosine phosphorylation of selected members of STAT family, STAT3 and STAT5 [32].

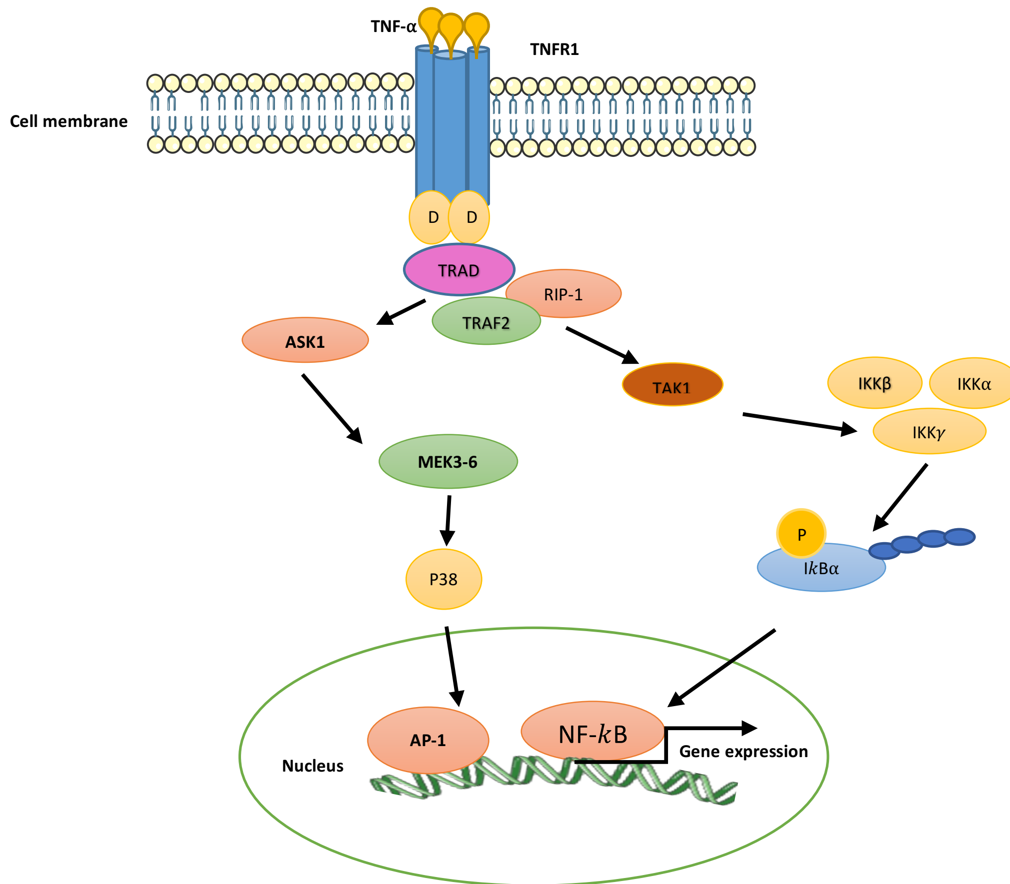

Figure 1.3- TNFR1 signal transduction pathway. Upon TNF-α binding to the receptor, the TRADD-TRAF2-RIP-1 complex activates kinase adaptor TAK1, which activates the IKK complex (IKKα, IKKβ and IKKγ) leading to phosphorylation and ubiquitin-mediated degradation of the IκB proteins and release NF-κB to the nucleus, where it binds to DNA promoter regions to induce gene expression. In addition, the TRAF-TRADD complex recruits ASK1 leading to phosphorylation of MEK3,6, which activates p38 MAPKs and thus (AP)-1, to facilitate gene transcription.

1.2.3- IL-1:

The IL-1 family consists of 11 members that share a similar β-barrel structure and bind to Ig-like receptors. IL-1α (IL-1F1) and IL-1β (IL-1F2) are produced by several cell types, including monocytes, macrophages, neutrophils and hepatocytes [33, 34]. The effect of IL-1 is mediated by the binding of ligands of the IL-1 family (IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RA)), to two major homology receptors (IL-1Rs, IL-1R1 and IL-1R2) [35]. Once IL-1β binds to IL-1R1 (which belongs to cytoplasmic TIR domain family), IL-1R1 forms a complex with receptor protein IL-1AcP, which is essential for downstream signal transduction [36]. IL-1AcP recruits proteins with kinase activity called Interleukin-1 receptor-associated kinases (IRAKs), (IRAK-1 and -2). MyD88 also associates with IL-1AcP, via its death domain to activate IRAK-2 [37, 38]. Activated IRAKs promote the polyubiquitination of TRAF-6, which in turn activates NF-κB-inducing kinase (NIK), which activates the IKK complex, leading to phosphorylation and degradation of IκB proteins, finally allowing NF-κB to translocate to the nucleus and initiate pro-inflammatory gene induction [39]. In addition, the activated TRAF6 recruits the protein kinase, TAK1, which stimulates MAP kinases that mediate JNK and p38 activation. Flowing IL-1β stimulation, TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein 2 (TAB2) also translocate from the membrane to the cytosol and functions as an adaptor protein, facilitating the interaction between TAK1 and TRAF6. This is a key step in activating TAK1, which leads to the induction of pro-inflammatory genes **(Fig. 1.4)** [40].

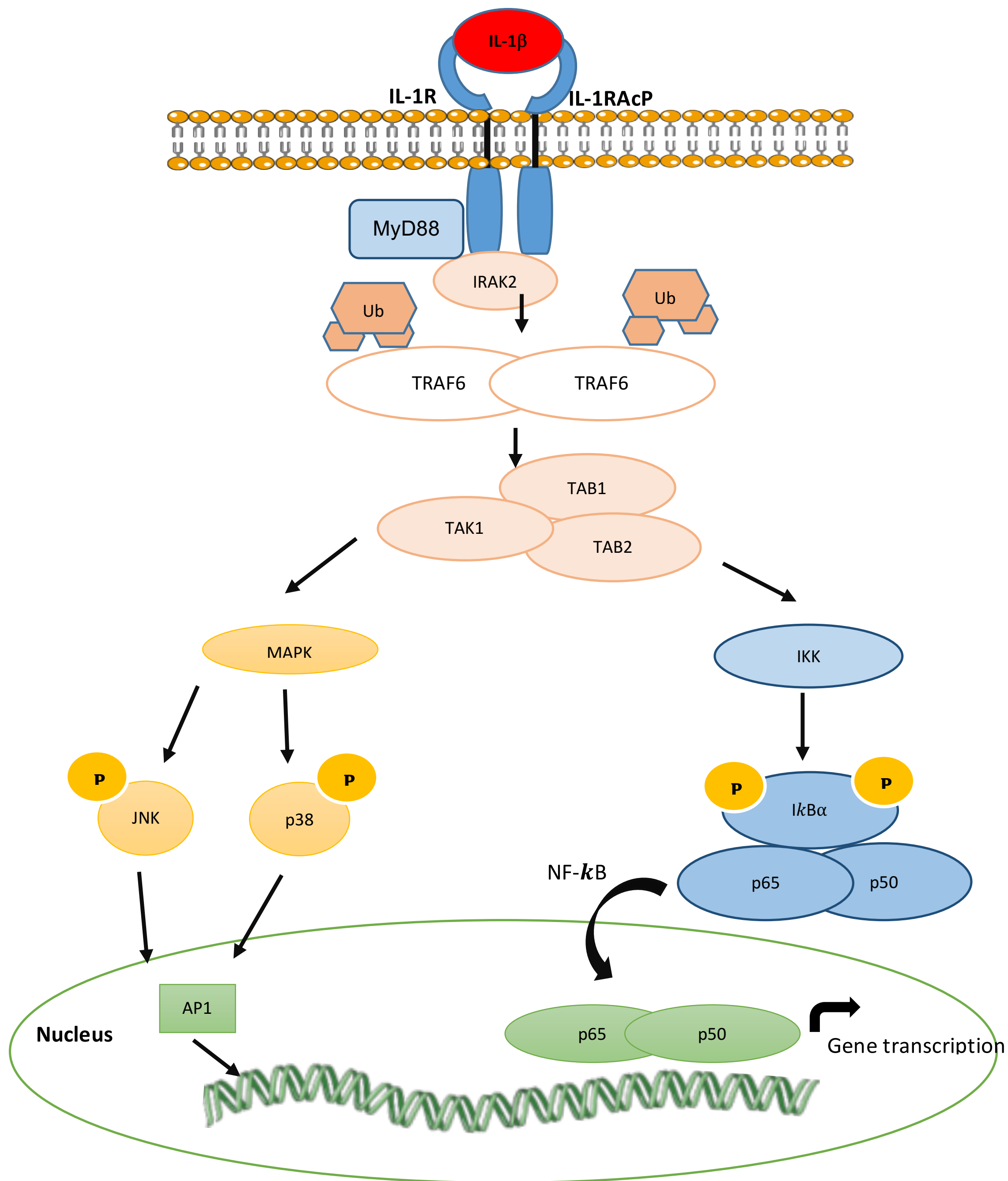

Figure 1.4- IL-1 signal transduction. IL-1β binds to the IL-1RI/IL-1RAcP complex, leading to the recruitment of MyD88 and IRAK proteins. Activation IRAK2 leads to subsequent activation of TRAF6, which interacts with TAK1 in a complex with TAB1 and TAB2, ultimately leading to the phosphorylation and degradation of IκBα, and the nuclear translocation of p65-p50 dimer. TRAF6 recruitment of TAK1 also stimulates MAPK, mediated JNK and p38 activation and leads to AP-1 activation.

IL-1 plays a major role in local and systemic inflammation and blocking IL-1 activity has revealed rapid and constant reduction in disease severity [41]. IL-1 has been linked to several diseases, including heart failure, rheumatological disease and gout arthritis [42, 43].

1.2.4- IL-6:

IL-6 is pleiotropic cytokine that modulates physiological events such as antigen-specific immune responses and inflammatory reactions, polarisation of naïve CD4+ T cells into effector T cells (Th1 and Th2) and promotion of plasma cells to produce immunoglobulin (Ig) [44, 45]. The biological activities of IL-6 are achieved through the IL-6 receptor (IL-6R) and signal transducer (gp130) chains. When IL-6 binds to the IL-6R, they form a complex that induces homodimerisation of gp130. Gp130 lacks an intrinsic kinase domain, instead it has a cytoplasmic domain that associates with JAK kinase to initiate the signalling cascade. Gp130 brings JAKs into close proximity. The activated JAKs (JAK1, JAK2 and Tyk2), phosphorylate and activate STAT proteins (homodimers (STAT3-STAT3) or heterodimers (STAT1-STAT3)), which consequently translocate to the nucleus and activate gene transcription. In addition, IL-6 triggers the MAPK pathway. Following IL-6 stimulation, gp130 recruits Src homology region 2-containing protein tyrosine phosphatase 2 (SHP-2) and which is rapidly phosphorylated by JAKs and interacts with growth-factor-receptor-bound protein 2 (Grb2) - son-of-sevenless (SOS) complex. SOS activates Ras through GDP/GTP exchange, which transmits the signal, leading to the activation of the ERK1/2 pathway. (**Fig. 1.5**). Additionally, IL-6 signalling is negatively regulated by SOCS proteins. The IL-6-IL-6R complex leads to the

activation of STAT3/STAT1, which induces the expression of both SOCS1 and SOCS3. SOCS1 directly binds to JAKs, via its SH2 domain and inhibits their catalytic activity, while SOCS3 inhibits the signalling via binding to the receptor complex (Tyr(P)-759 peptide of gp130) (**Fig. 1.5**) [46, 47]. IL-6 is an essential regulator of the immune response, therefore, IL-6 overexpression results in enhanced inflammation, but can also cause disease, such as systemic sclerosis [48] and rheumatoid arthritis [49].

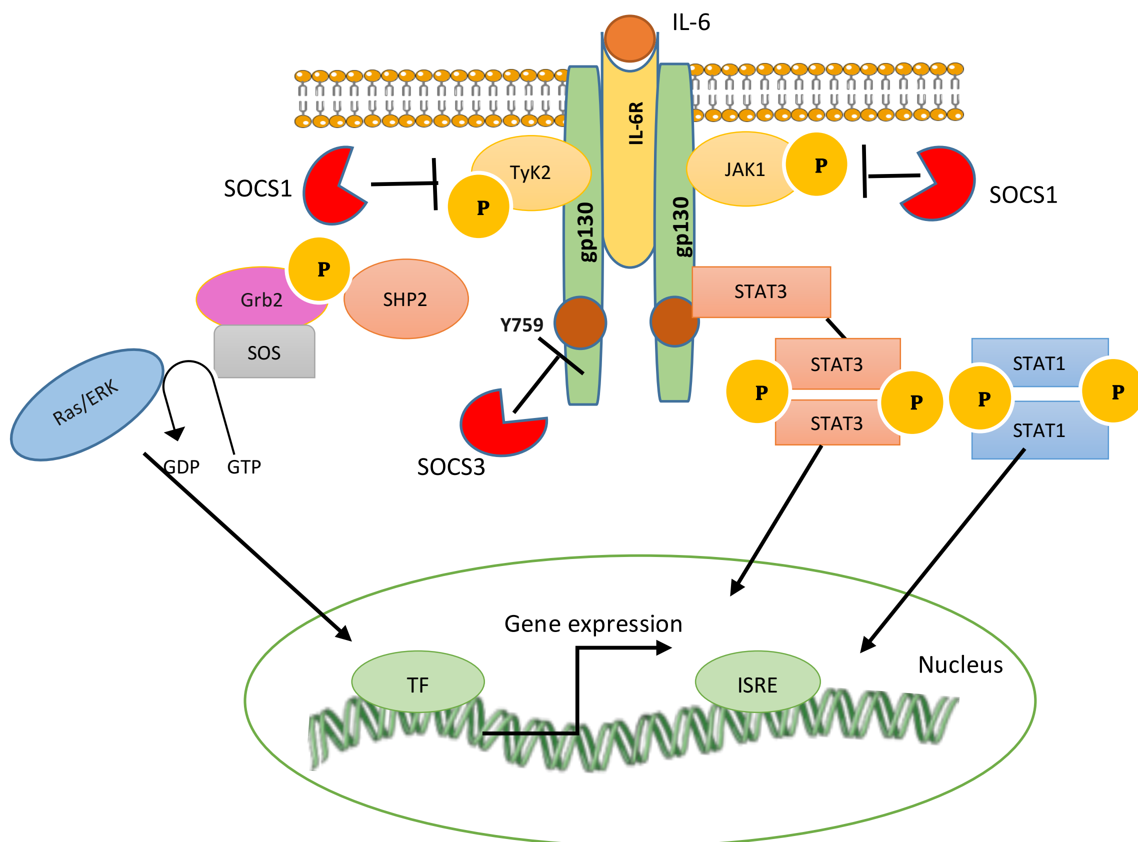

Figure 1.5- IL-6 signal transduction pathways. IL-6 binding to its IL-6R chain leads to homodimerisation of the gp130 receptors chain, which leads to JAKs phosphorylation. This then promotes the recruitment of STAT3 and STAT1, which in turn, induces the expression of SOCS3 and SOCS1. These SOCS inhibit signal transduction via binding to Tyr759 (SOCS3) or via direct binding to the JAKs (SOCS1). The gp130-mediated signals also activate the Ras/ERK pathway. Gp130 recruits SHP-2, which is rapidly phosphorylated by JAKs and

interacts with the Grb2-SOS complex. SOS activates Ras through GDP/GTP exchange, which transmits the signal, leading to the activation and translocation of ERK1/2 to the nucleus, where they induce inflammatory gene expression.

1.2.5- IFNs:

The IFN family plays a major role in activating anti-viral responses and immunomodulatory activities. Type I IFN family consists of IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. The IFN-α/β receptor (IFNAR) consists of two subunits, IFNAR1 and INFAR2 [50]. There is only one Type II IFN, IFN-γ; which binds to different receptors, IFN-γR1 and IFN-γR2 [51]. IFN-α and IFN-β are the best characterised type I IFNs, and are well known for their anti-viral roles. These IFNs induce the expression of hundreds of IFN-stimulated genes (ISGs), that have numerous actions, from directly inhibiting viral replication to activating other immune cells [52, 53]. Several PRRs recognise viral PAMPs; for example; TLR3 recognises viral dsRNA and immediately homo-dimerises, allowing interaction of its cytoplasmic TIR domain to the TRIF adaptor protein. TLR3 initiates signalling through the MyD88-independant pathway using the adaptor protein TRIF, which interacts with TRAF3 to activate TBK1 (and the non-canonical NF-κB pathway). Subsequently, these kinases phosphorylate interferon regulatory factor-3 (IRF-3), to form a dimer, which translocates to the nucleus, where it induces IFN-β expression **(Fig. 1.6)** [54, 55]. In contrast, TLR7/8 and 9 recruit MyD88 to form a complex with IRAK-4-TRAF6 which activates NF-KB and AP-1. Also the IRAK-1-TRAF6-IKK complex allows for the activation of IRF-7, which translocates to the nucleus and regulates the expression of IFN-α [56, 57].

IFN-β binding leads to dimerisation of IFNAR1 and IFNAR2, which cause auto-phosphorylation of the JAK1 and Tyk2 [32], which, in turn leads to phosphorylation of the receptors, providing docking sites for cytoplasmic STATs. STAT1 and STAT2 bind the receptor and themselves become phosphorylated, leading to their dissociation from the receptor, the STAT1/2 dimer associates with IRF-9, to form IFN-stimulated gene factor 3 (ISGF3). The ISGF3 complex translocates to the nucleus and binds the IFN- stimulated response element (ISRE) promoter site, leading to the induction of hundreds of ISGs, such as IFN- α and more IFN-β **(Fig.1.6)** [58].

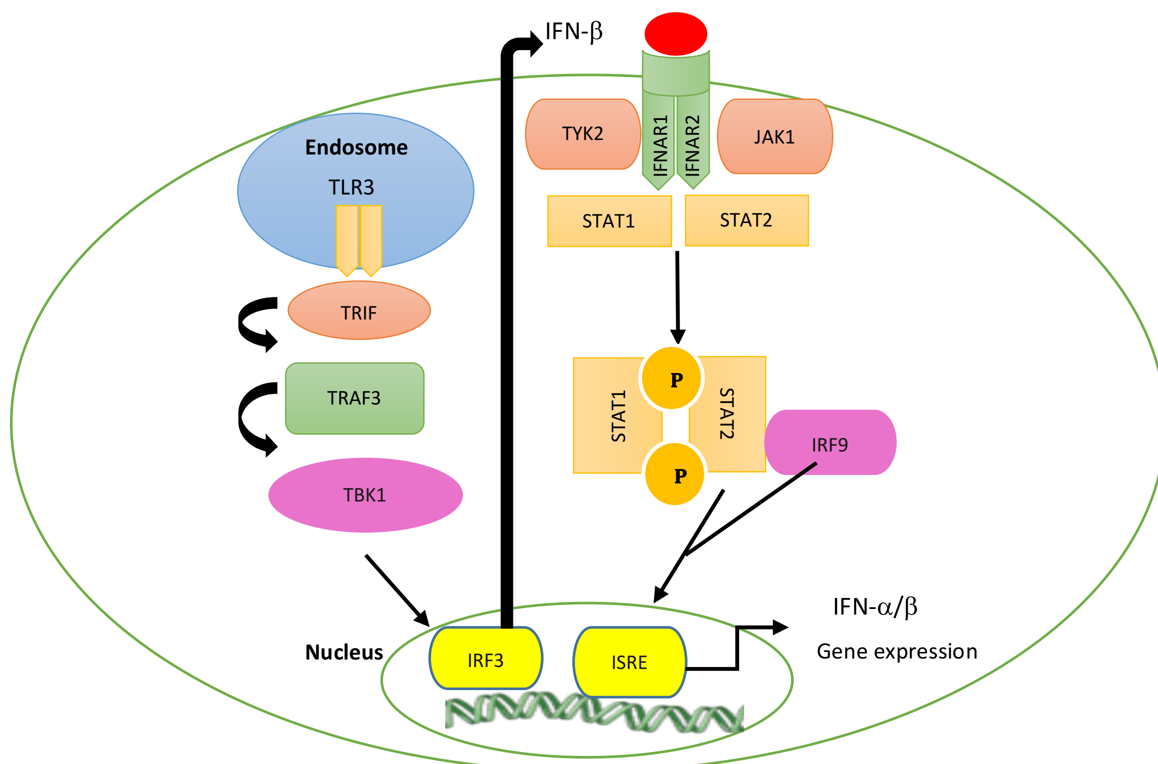

Figure 1.6- Type I IFN induction and signalling. TLR3 signals upon recognition of dsRNA, which results in TL3 homodimer, allowing interaction of its cytoplasmic TIR domain to the TRIF3 adaptor protein. TRAF3 activates TBK1 (and the non-canonical NF-κB pathway). Subsequently, these kinases phosphorylate interferon regulatory factor-3 (IRF-3), to form a dimer, which translocates to the nucleus, where it induces IFN-β expression. IFN-β binds to its receptor IFNAR1/2

on the cell surface, activates JAK1/TyK2, which phosphorylates the receptor and recruits STAT1 and STAT2, which when activated dissociate and dimerise. The heterodimer then complexes with IRF9 to form the ISGF3 complex which binds to ISRE promoter regions and induces the transcription of ISGs (producing IFN-α/β).

Type I IFNs play a critical role in many innate and adaptive immunological functions. IFN-α/β signalling can activate DCs, which act in an autocrine manner to stimulate T cells (enhancing proliferation of CD4+ and CD8+ T cells and subsequent IFN-γ production) [59, 60]. Indeed, the upregulation of other cytokines and chemokines, including IL-1, IL-2, IL-8, TNF-α and GM-CSF can also be regulated directly by the IFN-α signal transduction cascade [61].

1.3- Intracellular signal transduction

1.3.1- NF-κB signalling:

The activation of the NF-κB transcription factor captured the attention of many researchers in different fields due to its ability to induce transcription of pro-inflammatory genes (including TNF-α, IL-1 and IL-6) and thus control a range of immune and inflammatory responses [62]. The NF-κB family consist of 5 members, p50/105, p52/100, p65 (RelA), c-Rel and RelB; all of which are characterised by an N terminal Rel homology domain, that regulates the homo- or hetero-dimer binding to specific DNA elements [63]. The NF-κB dimers are normally found in the cytoplasm of non-activated cells sequestered by a family of inhibitory proteins called IκBs [64]. There are two main signalling pathways involved in the activation of NF-κB, the canonical and non-canonical (alternative) pathways **(Fig. 1.7)** [64]. The

canonical NF-κB pathway depends on the degradation of IκB by the IKK complex, (which compose of IKKα, IKKβ and regulatory subunit named NF-κB essential modulator (NEMO) or IKK-γ). The IKK complex is activated in response to different stimuli by various receptors, including PRRs, cytokine receptors, T cell receptors (TCRs) and B cell receptors [65]. Upon activation, IKK phosphorylates IκB-α on two N-terminal residues, targeting it for degradation, thereby releasing NF-κB (including RelA/p50), which translocate to the nucleus and binds to DNA promoter sites, thus regulating gene expression [66]. In contrast, the non-canonical NF-κB pathway is activated selectively by a specific group of stimuli, such as the TNFR superfamily, including TNFR-2 [67]. There are two essential components which participate in mediating the non-canonical NF-κB pathway: NIK and IKKα. NIK, a MAP3K, functions through activation of IKKα and facilitates the binding of IKKα to its substrate p100 [68]. The p100 complex contains an IKKα phosphorylation site on its C- and N- terminal, which is essential for NIK- induced ubiquitination and processing of p100. The degradation of p100 leads to the release of p52 which binds to RelB to form an active dimer. This active dimer (p52-RelB) translocates to the nucleus and induces the expression of non-canonical NF-κB genes (such as Bcl-2), which are involved in B cell survival [69].

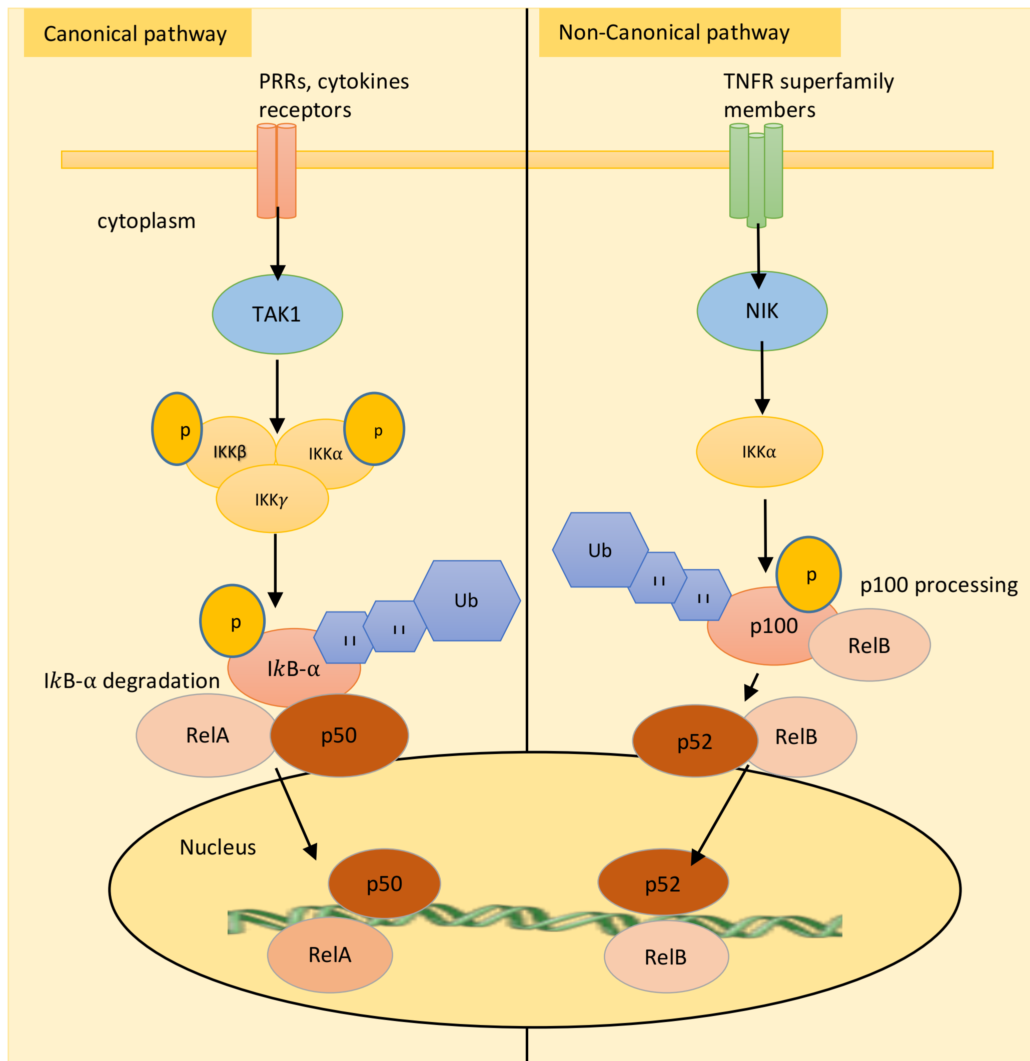

Figure 1.7- Canonical and non-canonical NF-κB pathways. The NF-κB family of transcription factors is activated by canonical and non-canonical signaling pathways, which differ in both signalling components and biological function. The canonical NF-κB pathway depends on the degradation of IκB by the IKK complex. The IKK complex phosphorylates IκB-α on two N-terminal residues, targeting it for degradation, thereby releasing p50-RelA dimer to the nucleus. While the non-canonical NF-κB pathway is mediated via NIK and IKKα. NIK, activates IKKα, which binds to its substrate p100. This leads to processing of p100, that results in the generation and subsequent DNA binding of active p52-RelB dimer, which translocates to the nucleus.

1.3.2- The JAK/STAT signalling pathway:

The JAK/STAT transduction pathway is an intracellular signalling cascade activated in response to extracellular ligands, such as cytokines and growth hormones, in order to regulate cell haematopoiesis and the immune response [70]. This activation requires binding of ligands to their receptors [71]. Once the ligand (such as a cytokine), is bound, this induces phosphorylation of the tyrosine kinase receptor. Depending on the ligand, phosphorylation of the ligand-receptor complex can be homo- or hetero-multimer. For example, IFN receptors form hetero-multimers with different receptor chains, making up the overall receptor complex. The Janus Kinase (JAK) family consists of four proteins, JAK1, JAK2, JAK3 and Tyk2. All JAKs contain a C-terminal tyrosine kinase regulatory domain and bind to membrane-bound receptors via their N-terminal domain [70]. Upon activation, two JAKs come into close proximity and phosphorylate each other, which then phosphorylate tyrosine kinase residues on the cytoplasmic region of the receptor, creating a phospho-tyrosine docking site for STAT proteins. STAT proteins are transcription factors that transmit signals from the receptor to the nucleus. There are 7 STAT proteins (STAT1, 2, 3, 4, 5a, 5b, 6). Once STATs bind to specific phospho-tyrosine residues on the receptors (the SH2 binding site), they become phosphorylated by JAKs, leading to their dissociation from the receptor and their dimerisation (through SH2-phosphotyrosine interaction). The STAT (homo- or hetero-)dimers travel to the nucleus, where they bind to specific regions of DNA (called STAT responsive elements), and promote gene transcription [72]. STATs contain the DNA-binding domain (DBD),

that mediates the binding to various DNA elements (such as the ISRE and the IFN-γ-activated site (GAS)-like response elements) [73] **(Fig. 1.8).**

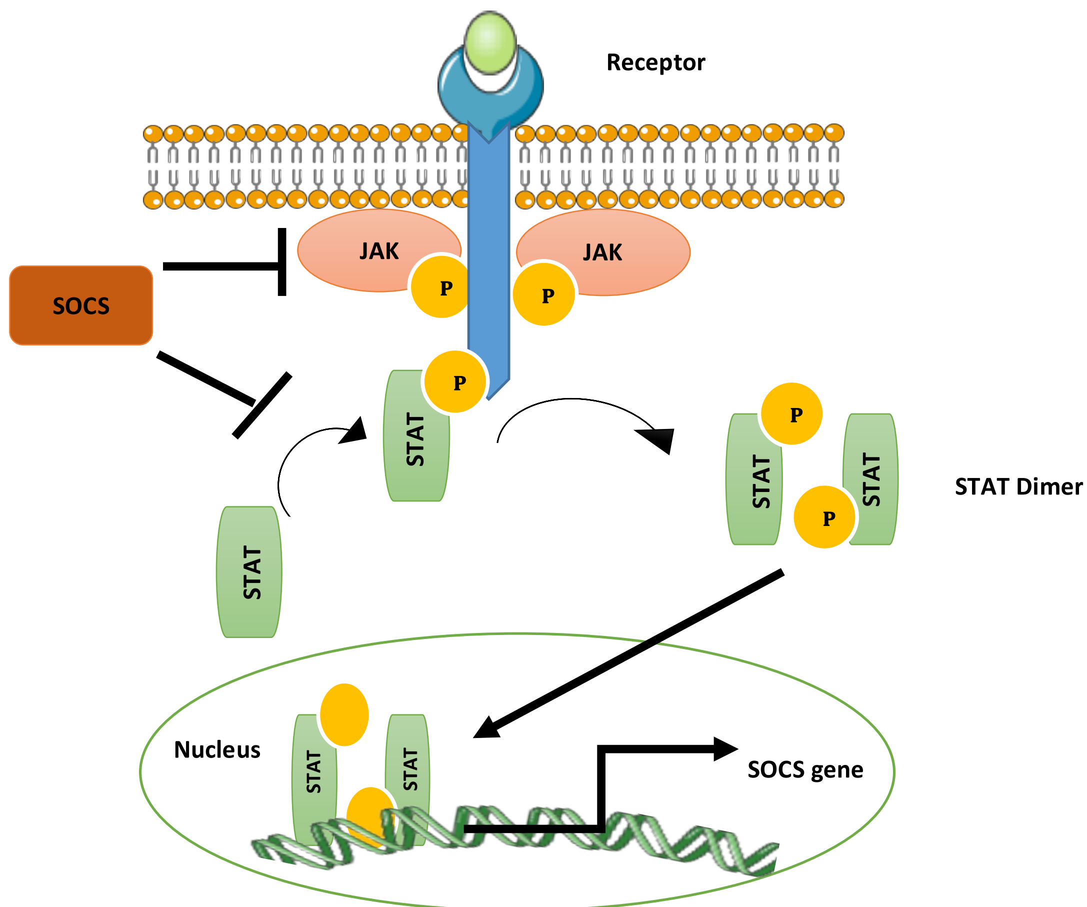

Figure 1.8- The JAK/STAT pathway. Ligands, such as cytokines, bind to their receptors, and activate JAKs. Activated JAKs then auto-and trans-phosphorylate each other, and phosphorylate tyrosine residues in the cytoplasmic domain of the receptor. STATs are then recruited to the receptor complex, phosphorylated by JAKs, form dimers, translocate into the nucleus, and initiate gene transcription, including that of SOCS, which acts in a negative feedback loop to inhibit signal transduction.

If this process is not tightly regulated, it could lead to a continuous, uncontrolled response, such as chronic inflammation, cytokine storm

or malignancy. Therefore, SOCS can function as inhibitors of the JAK/STAT signalling pathway [74].

1.4- Suppressor of cytokine signaling:

SOCS proteins have been shown to play vital roles in regulating innate and adaptive immune responses, through their negative regulation of JAK/STAT signaling [75]. The SOCS family are intracellular proteins, consisting of 8 members (CIS and SOCS1-7). SOCS proteins are induced in response to cytokines and act in classical negative-feedback loop [76].

1.4.1- The structure and function of SOCS:

The fundamental structure of all SOCS proteins is a central Src homology 2 domain (SH2) domain and a C-terminal SOCS box domain **(Fig. 1.9).** The SH2 is a small modular section, that binds particularly to the tyrosine-phosphorylated site on proteins [77]. The N- and C-extended SH2 domains (N-ESS and C-ESS) are critical regions for SOCS phospho-tyrosine binding. The N-ESS contains a 15-residue α-helix and the C-ESS contains 35 amino acid residues [78]. The SH2 domain of SOCS1 directly binds to the activated JAK loop while the SH2 domain of CIS, SOCS2 and SOCS3 interacts with phosphorylated tyrosine on activated cytokine receptors. For example the SOCS3 SH2 domain binds to the cytokine receptor subunit gp130 [79, 80].

The N-terminal domains of SOCS have varying amino acid lengths, CIS and SOCS1-3 tend to have shorter N-terminals while SOCS 4-7 have a longer N- terminal domains [76]. SOCS1 and SOCS3 share a common feature in their N-terminal, a kinase inhibitory region (KIR),

which allows them to directly inhibit cytokine receptor-associated JAKs [81]. Although the N-terminal of SOCS4-7 remain poorly understood [82], a study has shown a unique conserved region called the JAK interaction region (JIR) in the N-terminal of SOCS5 which is expected to inhibit JAK directly [83]. The SOCS6 N-terminal domain is involved in its transport to the nucleus which depends on 1-210 amino acid motifs that contain an undefined domain [84].

The SOCS-box is located in the SOCS C-terminus, consisting of two distinct motifs: the N-terminal half of the SOCS box is responsible for Elongin B/C binding, whilst the C-terminal portion encodes a Cul-box which interacts with Cullin family members, such as Cullin 5 [85, 86]. Cullin proteins then bind an additional protein, a RING-finger protein such as Ring- Box protein 2 (Rbx2) [86]. This complex has E3 ubiquitin ligase activity, which results in poly-ubiquitination and proteasomal degradation of SOCS binding proteins [75]. SOCS4-7 were originally recognised by their conserved SOCS box, which is an adaptor motif comprising three helices that interact with the substrate domain, such as an SH2 domain, within the ubiquitin ligase components Elongin B/C [75]

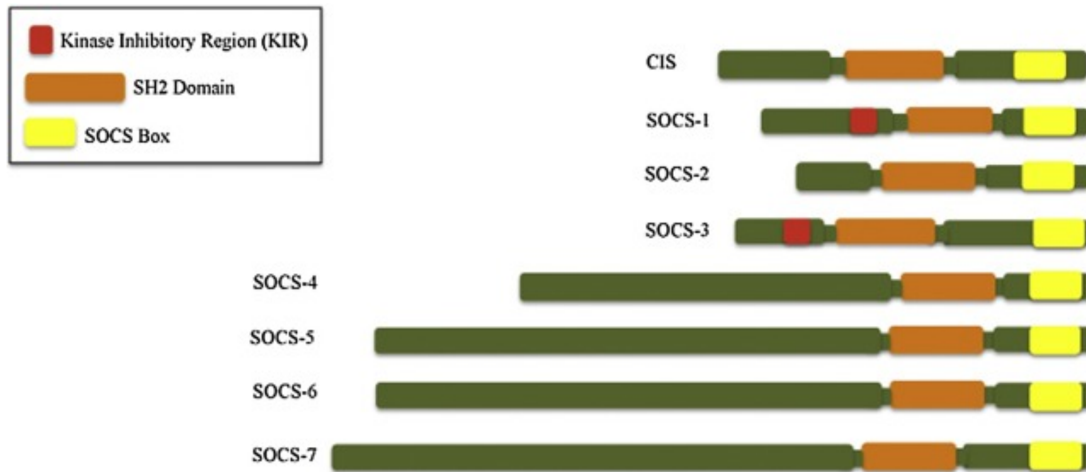

Figure 1.9- The structure of SOCS proteins. All SOCS proteins members consist of conserved SH2 and SOCS box domains. Some SOCS members (SOCS1 and SOCS3) have kinase inhibitory region (KIR) located in the N-terminal domain [87].

SOCS proteins are all able to regulate receptor signalling via targeted proteasomal degradation [88]. SOCS1 and SOCS3 can regulate signalling via directly binding of their KIR domain to the JAK catalytic domain, which blocks JAK kinase activity [89]. In addition, some SOCS can exert their regulatory function, such as CIS, SOC2 and SOCS3 by binding to the phosphor-tyrosine residues on the receptors, thereby obstructing the binding site for STAT, which effectively inhibits further signal transduction **(Fig. 1.10)** [90-92].

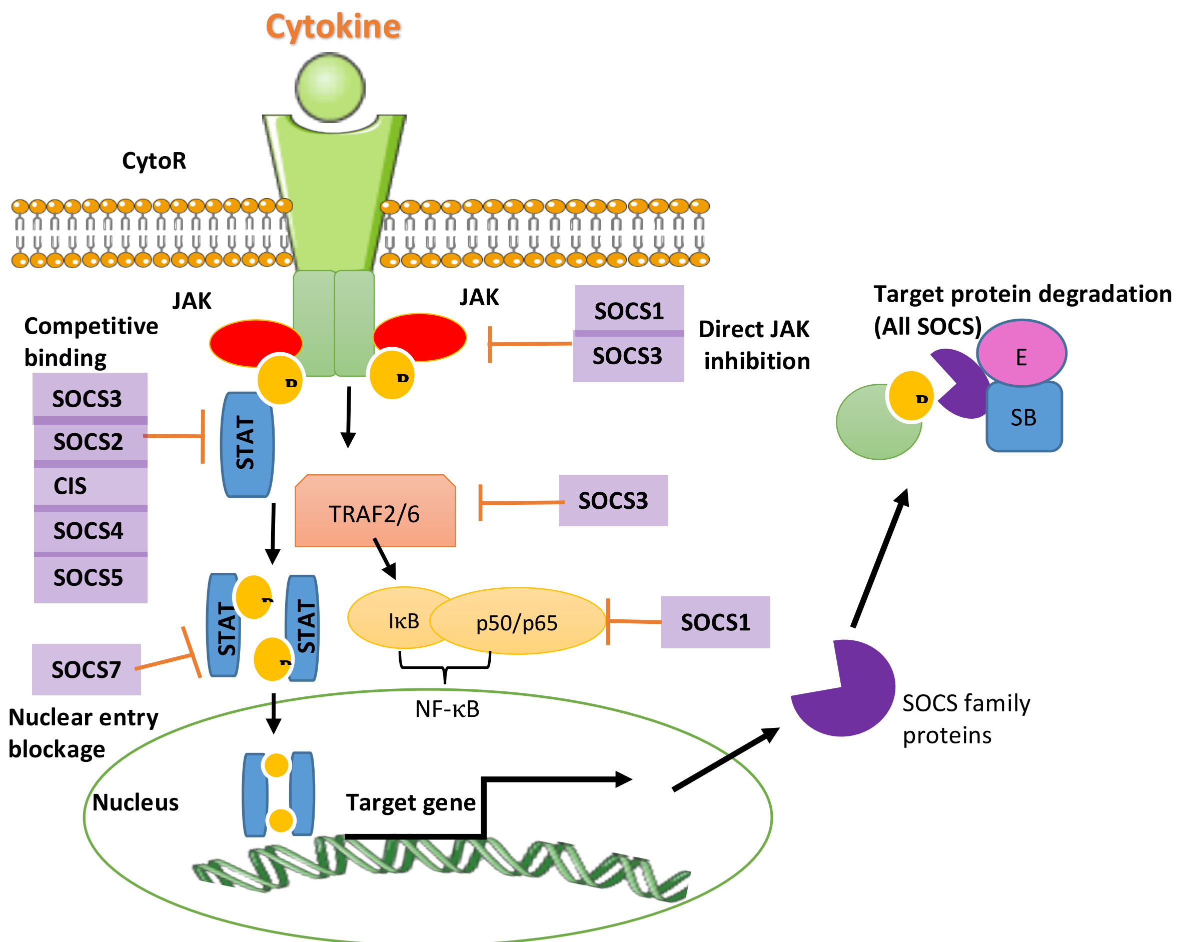

Figure 1.10- Schematic diagram showing SOCS mechanism of action. SOCS are capable of regulating receptor signaling, thus creating a negative feedback loop. Individual SOCS proteins negatively regulate signaling by several mechanisms: 1) degradation of receptors and/or associated proteins via the proteasome; 2) inhibition of JAK tyrosine kinase activity and 3) competition for receptor phospho-tyrosine residues, thereby blocking other signalling molecules from binding.

1.4.2- CIS:

Cytokine Inducible SH2 containing protein (CIS), was the first SOCS family member identified. CIS has an SH2 domain which associates with the tyrosine phosphorylated erythropoietin (EPO) receptor and tyrosine phosphorylated β-chain of the IL-3 receptor [93]. The expression of CIS is induced by IL-2, IL-4, granulocyte-macrophage colony-stimulating factor (GM- CSF) and EPO, which are mediators of STAT5 activation [94]. The mechanism of STAT5 inhibition occurs via direct binding of the CIS SH2 domain to the phosphorylated Tyr-401 of the EPO receptor (an essential residue for STAT5 activation), thus leading to the suppression of STAT5 activation [95].

1.4.3- SOCS1:

SOCS1 expression is induced by numerous cytokines, such as IL-4 and IL-6, which signal via activation of STAT6 and stimulate SOCS1 expression by binding to GAS motifs [96]. The SOCS1 KIR motif facilitates the direct inhibition of JAKs (JAK2 and Tyk2), by binding to their activation loops. Indeed, using the experimental allergic encephalomyelitis model, SOCS1 has been shown to inhibit IL-17 production using its KIR domain [97]. SOCS1 is rapidly induced by LPS, via TLR4 signalling, and it negatively regulates LPS-mediated NF-κB activation [98]. In addition to JAK/STAT and NF-κB, SOCS1 can inhibit other signalling cascades, such as the TNF-α-induced p38 MAPK pathway [99].

1.4.4- SOCS2:

SOCS2 shares a homology sequence with CIS and similarly functions by negatively regulating cytokines [95]. Growth Hormone (GH)

signalling is inhibited by SOCS1 and SOCS3. However, SOCS2 has a dual effect through inhibition of GH signal transduction and also restoration of GH signaling, via inhibition of other SOCS proteins [100]. SOCS2 enhances IL-2 and IL-3 signalling via reduction of SOCS3 protein expression. SOCS2 does this via binding to SOCS3 and recruiting Elongin B/C, thus forming an E3 ligase complex, which targets SOCS3 for proteasome-dependent degradation [101]. Furthermore, SOCS2 can block CIS interaction with leptin receptor (LR), by interfering with CIS binding position on the receptor (Tyr-1077 motif) and thereby restricting STAT5 recruitment and its signal transduction [102].

1.4.5- SOCS3:

SOCS3 is a key regulator of JAK/STAT signalling, through its ability to directly inhibit JAK1, JAK2 and Tyk2 catalytic domains, this is achieved via an SH2 domain, known as KIR [103]. SOCS3 regulates a spectrum of cytokines, and is well known to control IL-6-mediated STAT3 activation through interacting with gp130 [104]. In addition, SOCS3 inhibits antiviral responses by regulating the IFN-mediated JAK/STAT pathway [105]. Previous research in our lab has shown that SOCS3 inhibits TNF-α- mediated IκBα degradation and subsequent pro-inflammatory cytokine production, whereby SOCS3 associates with TRAF2 and inhibits downstream NF-κB activation. This inhibits the induction of pro-inflammatory genes, such as IL-8 [106]. Moreover, SOCS3 is reported to inhibit IL-1 signalling, via TRAF6 which prevents TAK1 activation and thereby subsequent NF-κB mediated gene induction [107].

1.4.6- SOCS4:

SOCS4 has an ESS region, located between the SOCS box and SH2 domain that stabilizes the central SH2 domain and a C-terminal SOCS box and mediates a conserved interaction with Elongin C [108]. SOCS4 is upregulated upon EGFR ligand binding, which stimulates the receptor's auto-phosphorylation. Tyr-1092 has been identified as a binding site for SOCS4 on the EGFR, which results in degradation and reduction in EGFR levels. Binding of SOCS4 to Tyr-1092 may also compete with STAT3 binding site. Together these findings strongly suggest a prominent role for SOCS4 in negatively regulating EGF signalling [109, 110]. **(Fig 1.11)**

SOCS4 deficient mice challenged with a virulent influenza strain (pathogenic H1N1 influenza virus), have enhanced weight loss, impaired viral clearance and a higher mortality rate. Even mice infected with a less virulent strain (H3N2 strain), had similar responses, indicating an important role for SOCS4 in the anti-viral response [111]. Compared to Wild Type (WT), SOCS4-/- mice also had increased levels of pro-inflammatory cytokines, including IL-6 and IFN-γ. The delay in viral clearance was linked to impaired trafficking of CD8+ T cells and TCR signalling, suggesting a role of SOCS4 in regulating TCR activation and signalling [111]. Indeed, the loss of in vivo protection against influenza reveals a significant role for SOCS4 in protection against viral infection.

Leukaemia inhibitory factor (LIF) regulates follicle development in mammals. Granulosa cells exposed to LIF activate JAK1/STAT3 signalling, which induces the expression of SOCS4, resulting in regulation of three target proteins (Cardiotrophin like Cytokine (CLC), Poly (rC) Binding Protein 1(PCBP1) and Cytosolic Malate

Dehydrogenase (MDH1)), which supresses the development of ovarian follicle. This identifies a role for SOCS4 in ovarian development [112].

SOCS4 has been identified as a tumour marker. One study on breast cancer tissue has reported the association of higher SOCS4 mRNA expression with early breast cancer tumours and highlighted a mechanism of JAK/STAT/SOCS4 interaction, identifying a role for SOCS4 in the development and progression of breast cancer [113].

1.4.7- SOCS5:

SOCS5 has partial homology with SOCS4, indicating possible functional similarity. Similar to SOCS4, SOCS5 plays a key role in negatively regulating EGFR signalling. EGF signalling promotes epithelial cell proliferation via direct STAT-SH2 binding or by means of SH2 adaptor proteins, Grb2 and Shc [114]. The EGFR family plays a role in the maintenance of tissue and is linked to the development of human diseases, such as chronic lung disease [115]. Following EGF stimulation, EGFR dimerize, which leads to the phosphorylation of the receptor tyrosine kinase (RTK)s, which then activates two major downstream signalling pathways: the MAPK signaling pathway and the JAK1-2/STAT signaling pathway. Many proteins play a role in the negative regulation of RTKs, such as SOCS proteins which can recruit an E3 ubiquitin ligase protein complex via their SOCS box, thus promoting ubiquitination and proteasomal degradation of target proteins. SOCS5 expression is increased in response to EGF and it subsequently promotes EGFR degradation. SOCS5's SH2 domain binds to the EGFR directly (via a phosphorylated tyrosine residue), or indirectly (via the phosphorylation of adaptor proteins, Grb2 and Shc). SOCS5 also uses its SOCS-box to recruit the Elongin B/C complex,

thereby forming an E3 ubiquitin ligase complex that mediates EGFR ubiquitination, marking it as target for proteasomal degradation, thereby preventing further signal transduction [110, 116]. **(Fig 1.11)**

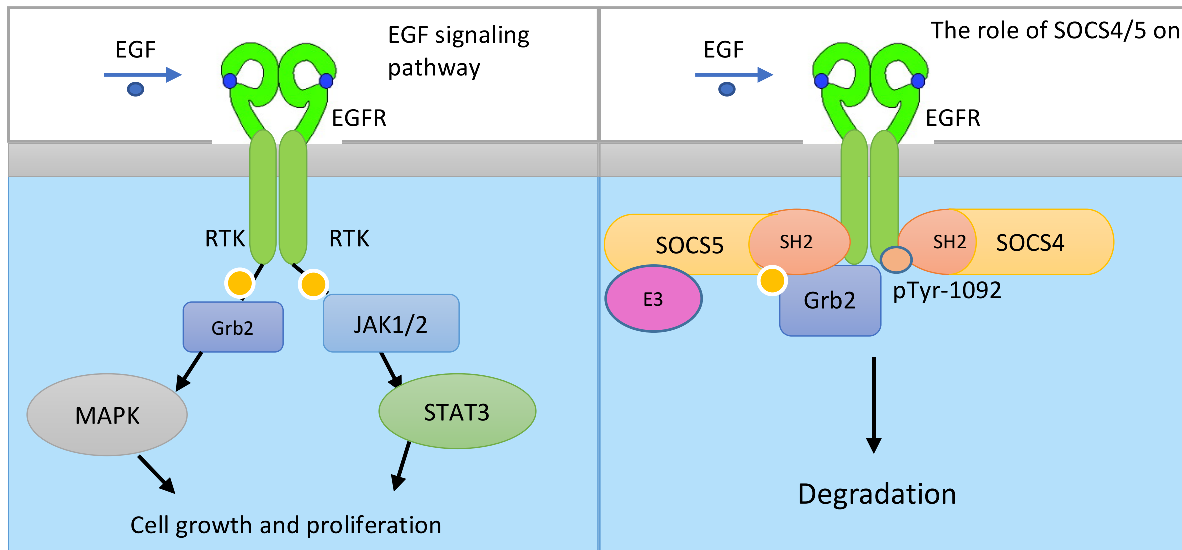

Figure 1.11. The EGF signaling pathway and its inhibition by SOCS4/5. Following EGF ligand stimulation, RTKs activate two major pathways (MAPK and JAK/STAT) to promote cell survival and growth. SOCS4 and SOCS5 have been shown to negatively regulate EGFR by targeting it for ubiquitin-mediated proteasomal degradation. SOCS5 utilizes its SH2 domain to bind directly to the receptor or indirectly via binding to the adapter proteins Grb2, or utilizing its SOCS-box to recruit the E3 ubiquitin ligase complex. SOCS4 binds to phosphorylated Tyr residue 1092 site of the receptors and thus inhibits JAKs and STAT3 phosphorylation.

SOCS5 has been shown to restrict the influenza virus through regulation of EGF-R signaling. The influenza virus activates the EGFR/ Phosphoinositide 3-kinases (PI3K) pathway and primary epithelial cells infected with influenza have been shown to have elevated levels of SOCS5 which in turn regulates EGFR/PI3K signalling. SOCS5-/- mice infected with influenza have increased viral

load in the lung, which indeed suggests the important role of SOCS5 in restricting viral replication [117].

SOCS5 has been reported to directly inhibit JAK phosphorylation using a different mechanism from SOCS2 and SOCS3. A unique region called the JAK inhibitory region (JIR), (located on the N-terminus of SOCS5), has the ability to bind and inhibit phosphylated JAK1 and JAK2, but the affinity of binding remains to be fully elucidated [118].

One study reported that SOCS5 controls the differentiation of T cells. The development of naïve T cells into Th2 cells is controlled by IL-4R JAK/STAT6 signalling. It has been shown that STAT6 activation after IL-4 stimulation is selectively impaired in Th1 cells, which is linked to the presence of SOCS5, which acts to inhibit the interaction between JAK1 and the IL-4R [119]. In contrast, another paper reported that SOCS5-/- mice showed no difference in the Th1/Th2 differentiation compared to WT control mice, indicating that SOCS5 has no *in vivo* impact upon the IL-4-mediated differentiation of T cells. Thus, this study concluded that despite SOCS5 being present in T cells, it is “non-essential” in regulating the development and the function of T naive cells in mice [120]. As there are conflicting reports regarding the role of SOCS5 in controlling T cell differentiation and since an appropriate T cell phenotype is instrumental in mounting an effective response to infection, the potential role for SOCS5 in controlling this process will be an important topic for future studies.

1.4.8- SOCS6:

SOCS6 is a suppressor of Insulin receptors (IR). The insulin resistance mechanism is mediated by cytokines, such as IL-1β, IL-6, GH, and TNF-α, which induce SOCS6 protein. The interaction of

SOCS6 with the IR was observed in human and rat hepatoma cells[121].

SOCS6 has also been reported to contribute to the suppression of tumours. c-KIT is a tyrosine kinase activated in response to stem cell factor (SCF). Its activity needs to be tightly regulated to ensure normal proliferation of hematopoietic cells, however, its over activity has been linked to tumour development. SOCS6 interacts with c-KIT's Tyr-568, via its SH2 domain, and inhibits signalling, which regulates cell growth. In addition, SOCS6 utilizes its SOCS-box domain to interact with Elongin B/C and Cul5/RBX2, which together mediate ubiquitination-mediated proteasomal degradation and thus regulates the c-KIT protein turnover [122]. SOCS6 has also been shown to have a regulatory role in acute myeloid leukemia (AML). Fms-related tyrosine kinase 3 (Flt3) is a ligand-dependent growth factor receptor and its mutation contributes to AML. SOCS6 binds to two phosphorylated sites of the Flt3 receptor (the pTyr-591 and pTyr-919), and thus targets the receptor for ubiquitination, internalisation and proteasomal degradation [123].

SOCS6 protein and mRNA are expressed at low levels in primary human colorectal cancer tissue, suggesting a potential role for SOCS6 in this cancer and highlighting it as a possible diagnostic marker [124]. Moreover, SOCS6 is thought to be involved in the recurrence of lung squamous cell carcinoma (SCC). The reduction of SOCS6 mRNA expression is linked with increased methylation of its gene, further indicating that loss of SOCS6 could be a useful prognostic biomarker of SCC [125].

1.4.9- SOCS7

Like all other SOCS proteins, the SH2 domain of SOCS7, binds to tyrosine phosphorylated kinases of activated receptors [126]. However, few studies have demonstrated the biological role of SOCS7. One report suggests a possible role for SOCS7 in inhibiting prolactin (PRL), GH, or leptin (LEP) signaling, via STAT5 and STAT3. SOCS7 blocks the activation of both STAT3 and STAT5 and their translocation to the nucleus, by binding directly to their phosphorylated residues [127].

Among the regulatory functions of SOCS7, one report demonstrates the involvement of SOCS7 in negatively regulating the Hepatocyte growth factor (HGF) pathway. HGF is a multifunctional cytokine and together with its receptors C-MET, they are critical mediators of breast cancer progression in breast cancer cells. SOCS7 gene suppression in breast cancer cells (MCF7) had shown amplification of HGF/C-MET growth and migrational signalling [128].

SOCS7 has also been reported to bind to insulin receptor substrate 1 (IRS1). SOCS7-deficient mice showed lower glucose levels and prolonged hypoglycemia during an insulin tolerance test and exhibited increased glucose clearance, suggesting that SOCS7 plays a role in regulating insulin signalling and thus glucose homeostasis [129].

In summary, several studies have confirmed that cytokine induced signalling pathways are negatively regulated by SOCS. While the induction and role of SOCS1-3 and CIS have been comprehensively studied, there is significantly less known about the role of SOCS4-7. Therefore, in a bid to further elucidate the role of this family of regulatory proteins, this project analyses the induction and regulatory

role of SOCS4 and SOCS5. Furthermore, since SOCS4 and SOCS5 have been both linked to the anti-viral and inflammatory immune responses we focused our investigations upon their role during IL-6, IL-1β, TNF-α (pro-inflammatory) and IFN-α (anti-viral) signaling.

1.5. Project Hypothesis and Aims:

Since SOCS4 and SOCS5 have been linked to the regulation of pro-inflammatory and anti-viral immunity [111, 130], we hypothesised that SOCS4 and 5 are induced by the pro-inflammatory cytokines IL-6, IL-1β and TNF-α, and the anti-viral cytokine, IFN-α. Furthermore, we also hypothesised that the action of both SOCS4 and SOCS5 may regulate the signal transduction of pro-inflammatory cytokines, such as IL-6-mediated STAT3 phosphorylation and TNF-α-mediated IκBα degradation.

In order to investigate these hypotheses, this project aimed to define the following research goals:

1. Determine the ability of pro-inflammatory (IL-6, IL-1β, TNF-α) and anti-viral (IFN-α) cytokines to induce SOCS4 and SOCS5 mRNA and protein expression

2. Confirm the expression of SOCS4 and SOCS5 plasmids in the HEK 293T cell line

3. Elucidate the role of SOCS4 and SOCS5 in the regulation of IL-6-mediated STAT3 activation **(Fig.1.12).**

4. Elucidate the role of SOCS4 and SOCS5 in TNF-α-mediated NF- κB signalling **(Fig.1.12).**

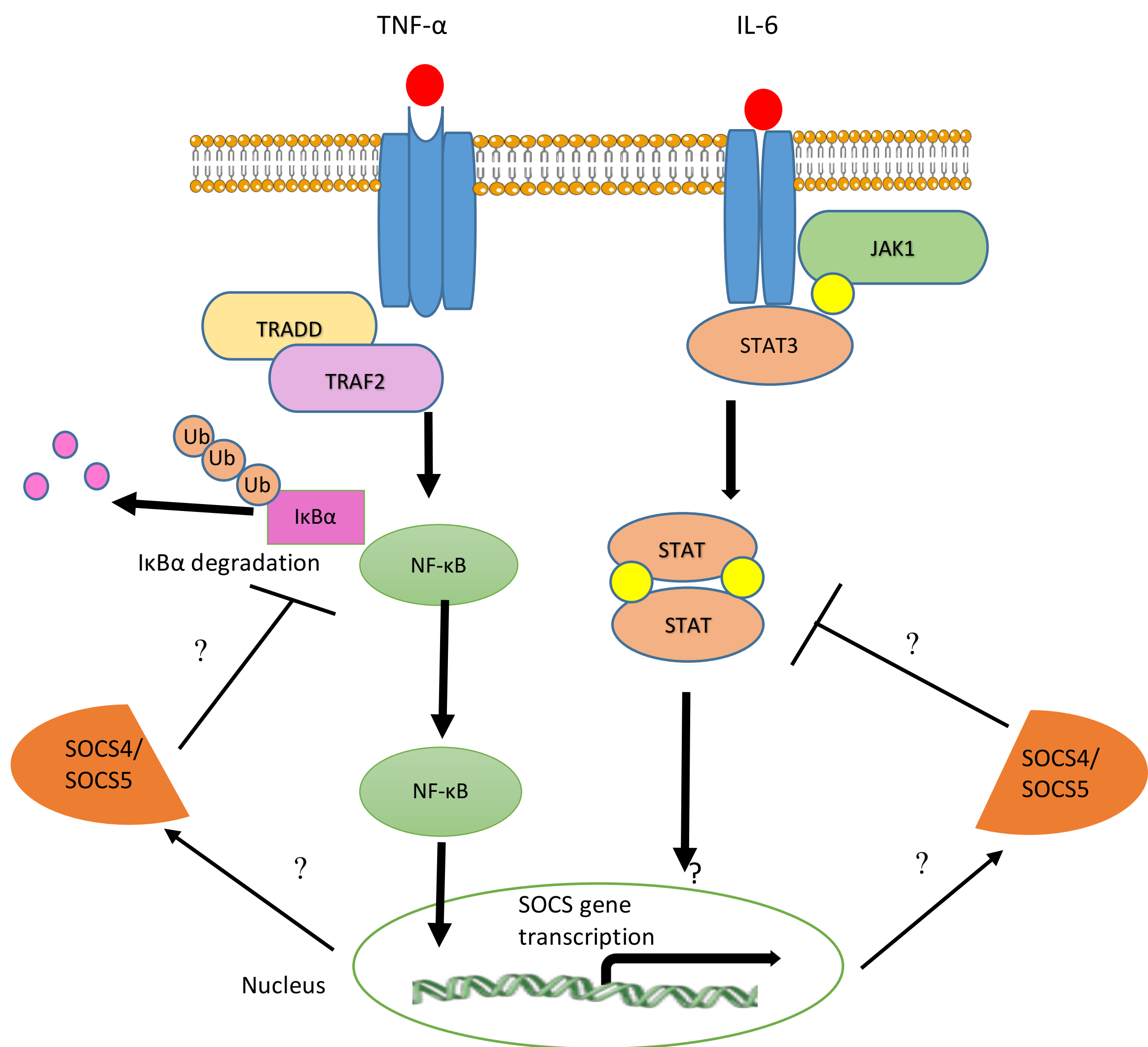

Figure 1.12. Schematic diagram detailing the research questions of this project: 1) Do pro-inflammatory cytokines induce SOCS4 and SOCS5 gene expression? and 2) Do these proteins act to regulate IL-6-mediated STAT3 phosphorylation and TNF-α-mediated NF- κB signalling?

Chapter 2
Materials and Methods

2.1- Transformation:

Constructs encoding SOCS4, SOCS5 or the Empty Vector (EV) control were transformed into bacterial cells (E. coli). 1µl of DNA plasmid was added to 5µl of Stellar Competent cells (Clontech). The cells were incubated on ice for 30min then heat shocked at 42°C for 45sec. Finally, the cells were incubated on ice for 2min. 1ml of LB broth was added to the cells, which were then incubated at 37°C with shaking (250 rpm) for 1h. Cells were then centrifuged at 3000g for 3 min. 800µl of supernatant was removed and cells were resuspended in the remaining 200µl and spread evenly on LB-Agar plates supplemented with ampicillin (50µg/ml) and incubated for overnight at 37°C.

2.2- DNA plasmid purification:

A single colony was chosen and transferred to 100ml LB broth supplemented with ampicillin (50µg/ml) and left overnight at 37°C with shaking. The DNA plasmids (SOCS4, SOCS5 and EV) were purified using QIAGEN HiSpeed® plasmid midi kit. Nanodrop Spectrophotometer was used to measure the DNA concentration.

2.3- Cell Culture:

HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin. The cells were maintained at 37°C in 5% CO2 and 95% humidity. The cells are passaged when they reached 80-90% confluency.

2.3.1 Transfection:

Cells were counted and seeded in 6 well plates at a density of 2.5x10^5cells/ml and incubated for 24h. 2μg of DNA plasmid (EV, SOCS4 and SOCS5) was transfected using 4μl of transfection reagent Lipofectamine TM2000 (INVITROGEN) per well. After 48h incubations, cells were stimulated with the cytokines of interest.

2.3.2 Stimulation:

Cells were seeded at a density of 2.5x10^5cells/ml in 6-well plates and incubated for 24h. Then the cells were rested in DMEM supplemented with 2% FBS for at least 2h. The cells were then stimulated with cytokines of interest **(Table 2.4)**. After a time course of stimulation, the cells were harvested for RNA or protein.

2.4. Real time- quantitative Polymerase Reaction (qRT-PCR).

2.4.1 RNA extraction.

Total cellular RNA was isolated by adding 500μl/well of TRI reagent (Sigma) after the cells were washed with cold PBS and incubated for 5min. 100μl of chloroform was then added to the solution which was incubated for 5min at Room Temperature and then centrifuged at 12,000g for 15min at 4°C. This resulted in three separated layers, the top which is RNA-containing aqueous layer, the middle layer (white) containing DNA and the pink bottom layer containing other organic material. 100μl of the RNA-containing layer was transferred to a new tube and 250μl of iso-2-propanol was added and incubated at RT for

10min followed by centrifugation at 12,000g for 10min at 4°C. The RNA pellet was washed with 500µl of 75% ethanol and then centrifuged at 7,500g for 5min at 4°C. after removing ethanol, the RNA pellet then re-suspended in 20µl of RNAse free water. The RNA was incubated at 60°C for 10min to aid solubilization and then placed on ice. The concentration of RNA was measured by NanoDrop® 2000 Spectrophotometer and stored at -20°C.

2.4.2 cDNA Synthesis.

cDNA was prepared from the extracted RNA by using the SensiFAST cDNA synthesis kit (Bioline). 500ng of RNA was added to DNAase/RNAase free water. The mix was heated at 70°C for 5min and then placed in ice for 2min. 1µl of 5x TransAmp Buffer and 0.25µl of reverse transcriptase enzyme were both added to the mix and the reaction was placed in PTC-2000 Peltier Thermocycler at 25°C for 10min (primer annealing), 42°C for 15min (reverse transcription) and 85°C for 5min (enzyme inactivation). Samples were then stored at -20°C.

2.4.3 qRT-PCR

10 µl of reaction mix per well consisting of 4µl of SYBER master mix reagent, 4µl of DNAasa/ RNAase free water, 500nM of both Forward and Reverse primers **(Table 2.1)** and 1µl of cDNA. The mix was added to a 96-well PCR plate and sealed tightly. The plate was then placed in CFX 96™ Real-Time System. The data was analyzed using Bio-Rad software.

*Cycle conditions of PCR:

cDNA was denatured at 95°C for 15min, followed by denaturation at

95°C for 30sec, primer annealing at 65°C for 1min and elongation at 72°C for 30sec. The cycle was repeated 40 times. All gene amplification was normalized to β-actin (**Table 2.1**).

The $2^{-\Delta\Delta ct}$ method was used to analyse gene expression, a ratio of gene relative to the housekeeping control was calculated by subtraction of the Ct values for reaction from the Ct values of the β-actin (endogenous control). The given ∆Ct value of the calibrator sample was then subtracted from the ∆Ct value of each sample. The resulting ∆∆Ct value is normalized using the formula: $2^{-\Delta\Delta ct}$ that represents the fold change in expression for a test sample relative to the calibrator sample for a particular gene.

2.5. Western Blot

2.5.1 Protein lysate.

Cells were lysed using 1x (RIPA) lysis buffer (5x RIPA lysis buffer, 1M tris(hydroxymethyl)aminomethane (Tris) pH 8.0, 5M NaCl, 20% v/v Triton X-100, 20% w/v SDS made up in dH_20), which is supplemented with protease and phosphatase inhibitors: Leupeptin (0.5µg/ml), Na_3VO_4 (1mM), PMSF (1mM) and DTT (1mM). after adding 1x RIPA buffer, the cells were lysed at 4°C for 30min, then centrifuged at 12,000g for 10min. The protein containing lysates were then transferred to new tubes and stored at -20 °C.

2.5.2 SDS-PAGE.

Discontinuous Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) was carried out using the BioRad Gel Electrophoresis system. Polyacrylamide gels were made by preparing a 10% or 12.5% resolving gel and a 5% stacking gel **(Table**

2.3). The lysate samples mixed with a 4x loading buffer then boiled at 100°C for 10min before being loaded to the gel. The gels were run at 80V for 10min until the samples had entered the resolving gel, then at 110V.

2.5.3 Transfer to PVDF.

The proteins were transferred to a Polyvinylidenedifluoride (PVDF) membrane. PVDF was activated in methanol and then soaked in 1x Transfer Buffer (25mM Tris-HCl pH8.0, 0.2M glycine, 20% methanol) before the gel was placed on the membrane. The proteins were transferred at 110 V for 90min. Membranes were blocked to prevent the non-specific binding of antibody in 5% w/v marvel (5% w/v powdered milk in 1XTBST) for 1h.

2.6- Primary and Secondary Antibodies.

Membranes were then incubated overnight at 4°C with primary antibody **(Table 2.2)** (diluted in either 5% w/v milk or 5% w/v BSA) (table 2). After washing the blots in 1x TBST three times (5-10 minutes each), immunoblots were incubated with anti-rabbit or anti-mouse antibodies **(Table 2.2),** which was prepared in 5% milk – TBST buffer, for an hour at room temperature. A solution of Enhanced Chemi-luminesence (BioRad) reagent was used in the detection of the immunoreactive bands and the blots were analyzed using Gel Doc Imaging System (Biorad).

2.7- Densitometry Analysis:

Image Lab™ software version 5.2.1 was used to analyse the blots. The selected bands were normalized to the loading control β-actin.

The fold change represented the ratio of protein to β-actin band intensity, relative to non-treated control which was normalised to 1. The statistical analysis was analysed by using prism (version 6).

2.8- Tables:

Gene Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Source of primer
SOCS4	CTTAGATCATTCCTGTGGGC	ATGCCACCTAAGGCTAAATC	Sigma
SOCS5	TACAGCAAGCAGTCAAAGCC	ACAGAGAAGAGGTAGTCCT C	Sigma
β-ACTIN	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	Sigma

Table 2.1- Forward and reverse primer sequences for qRT-PCR.

Primary Antibody	Description	species	Company	Probing dilution	Secondary Antibody
SOCS4	Rabbit pAb	Rabbit IgG	GeneTex	1:500	Anti-Rabbit HRP (Invitrogen)
SOCS5	Mouse mAb	Mouse IgG	Santa Cruz	1;1000	Anti-Mouse HRP (Invitrogen)

FLAG	Mouse mAb	Mouse IgG	Sigma-Aldrich	1:2000	Anti-Mouse HRP (Invitrogen)
TOTAL STAT3	Mouse mAb	Mouse	Brennan & company	1:1000	Anti-Mouse HRP (Invitrogen)
Phospho STAT3	Rabbit pAb Phosphorylation site Tyr705	Rabbit	Cell Signaling	1:1000	Anti-Rabbit HRP (Invitrogen)
IkBa	Gift	Mouse	N/A	1:2000	Anti-Mouse HRP (Invitrogen)
β-actin	Mouse mAb	Mouse IgG2a	Sigma-Aldrich	1:2000	Anti-Mouse HRP (Invitrogen)

Table2.2- Antibodies for Western Blotting.

Resolving Gel:

Gel percentage (based on molecular Weight of protein used in this study)	10% (40-120)	12.5% (15-40)
Acrylamide	**5ml**	**6.25ml**
dH_2O	**4.8ml**	**3.55ml**
1.5 M Tris-HCL pH (8.8)	**5.6ml**	**5.6ml**
10% SDS	**150µl**	**150µl**

10% APS	75µl	75µl
TEMED	15µl	15µl

Stacking Gel:

Acrylamide	1ml
dH2O	4.61ml
1 M Tris-HCL (pH 6.8)	750µl
10% SDS	30µl
10% APS	60µl
TEMED	12µl

Table 2.3- Component of resolving and stacking gels.

Cytokines	Concentration /ml Used	Company
Human TNF- α	20ng/ml	Peprotech
Human IL-6	25ng/ml	Peprotech
Human IL-1β	10ng/ml	Peprotech
IFN-α 2a human	1000Unit/ml	Sigma-Aldrich

Table 2.4- Cytokines.

Chapter 3

The cytokine-mediated regulation of SOCS4 and its role in IL-6 and TNF-α signal transduction

3.1. SOCS4 mRNA and protein are regulated by pro-inflammatory (IL-6, IL-1β, TNF-α) and anti-viral (IFN-α) cytokines

Little is known about the expression of SOCS4, beyond its induction by EGF [110] and LIF [112]. However, since SOCS4-/- mice, challenged with influenza, are unable to control infection and have excessively high levels of pro-inflammatory cytokines, that are linked to their death [111], we hypothesised that SOCS4 has a role in both inflammatory and anti-viral signalling. To investigate this hypothesis, we first analysed the effect of key pro-inflammatory cytokines, IL-6, IL-1β, TNF-α, and the antiviral cytokine, IFN-α, upon SOCS4 mRNA and protein expression

3.2- Investigating the effect of pro-inflammatory cytokines upon SOCS4 expression.

3.2.1- IL-6 induces the expression of SOCS4 mRNA and protein. Firstly, we analysed if SOCS4 mRNA levels were affected by IL-6 stimulation. HEK 293T cells were treated with IL-6 (25ng/ml), over a time course of 0, 1, 2, 3, 4, 6 and 24h, before harvesting total RNA via the phenol-chloroform method. SOCS4 mRNA levels were quantified relative to the housekeeping gene, β-actin, and were analysed by qRT-PCR. While not significant, SOCS4 mRNA levels fluctuated over the first 6h of IL-6 treatment, but were significantly upregulated after 24h, with a mean fold induction of 2.7 ($p=0.01$) (**Fig. 3.1)**.

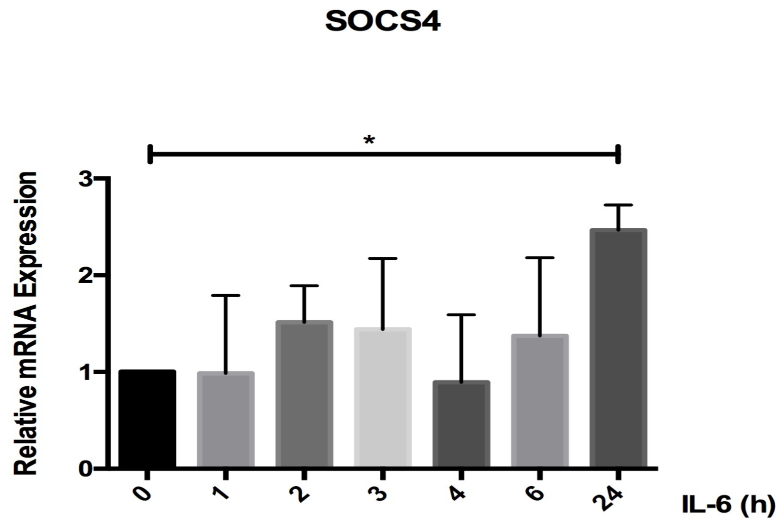

Figure 3.1: IL-6 induces SOCS4 mRNA. HEK293T cells were treated with IL-6 (25ng/ml), after being rested for 2h in DMEM supplemented with 2% FBS. SOCS4 mRNA expression was normalised to the housekeeping gene, β-actin. IL-6 treated samples were displayed relative to the untreated ("0") control, which was normalised to 1. The results are displayed as the mean (+/- SD) of three independent experiments and analysed by paired t-test *p<0.05.

Having observed a significant increase of SOCS4 mRNA after IL-6 stimulation, next, we investigated if this mRNA upregulation corresponded to an increase in SOCS4 protein. HEK 293T cells were treated with 25ng/ml of IL-6 for 0, 2, 4, 6 and 24h. The cells were then harvested and lysates were analysed by SDS-PAGE, before Western blots were probed for SOCS4 and β-actin antibodies. Fig. 3.2B shows that SOCS4 protein was endogenously expressed at low levels, but protein levels immediately increased after 2h of IL-6 treatment. Indeed, as we observed at the mRNA level **(Fig. 3.1),** SOCS4 protein levels continued to increase and were highest at 24h. While not statistically significant upon densitometric analysis of three independent experiments, we observed a continual rise in SOCS4 protein expression over the 24h time course; most noticeably with a

2.2-fold increase after 6h IL-6 stimulation, as well as 2.5-fold increase after 24h **(Fig. 3.2A)**.

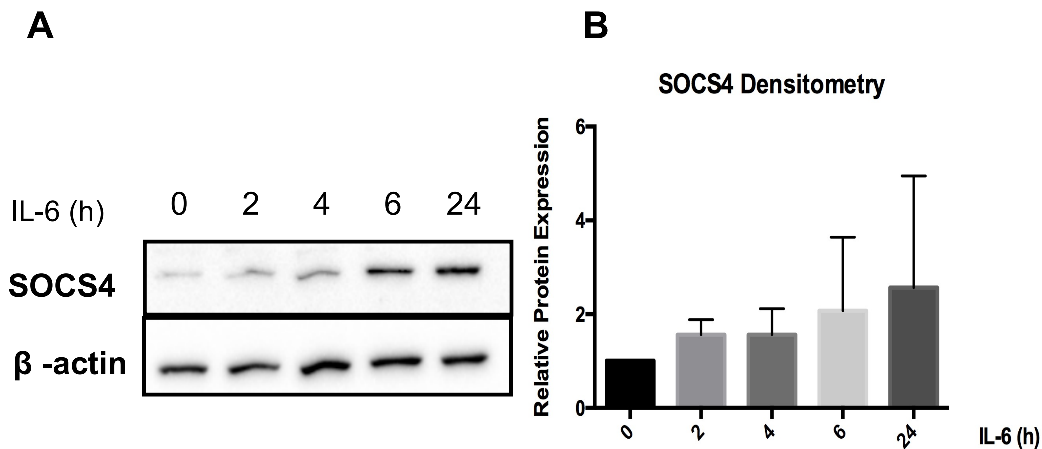

Figure 3.2: IL-6 induces SOCS4 protein. (A) HEK293T cells were treated with IL-6 (25ng/ml), after being rested for 2h in DMEM supplemented with 2% FBS and lysates were analysed by SDS-PAGE and Immunoblotted for SOCS4 and β-actin. **(B)** Densitometric analysis of three independent experiments was performed by using Image lab software and the statistical analysis was performed by using prism. Graph is the mean +/- SD of three independent experiments, SOCS4 compared to the β-actin and treated sample values are displayed relative to the untreated (0) sample, which was normalised to 1.

3.2.2- IL-1β induces SOCS4 mRNA and protein.

Having discovered that IL-6 regulates the expression of SOCS-4 mRNA and protein, we next sought to determine whether SOCS4 expression was also controlled by another key inflammatory cytokine, IL-1β. HEK 293T cells were treated with IL-1β (10ng/ml) over a 24h time course (0, 1, 2, 3, 4, 6 and 24h). Cells were harvested using the Phenol-chloroform method and the isolated RNA was reverse transcribed to cDNA, which was used as template for qRT-PCR. The graph shows mean mRNA levels immediately increased, rising to 2.4-

fold after 1h and 4.9-fold after 2h; however, there was a statistically significant 3.4-fold upregulation of SOCS4 mRNA after 4h of IL-1β treatment (p=0.03 by paired t-test) **(Fig. 3.3).**

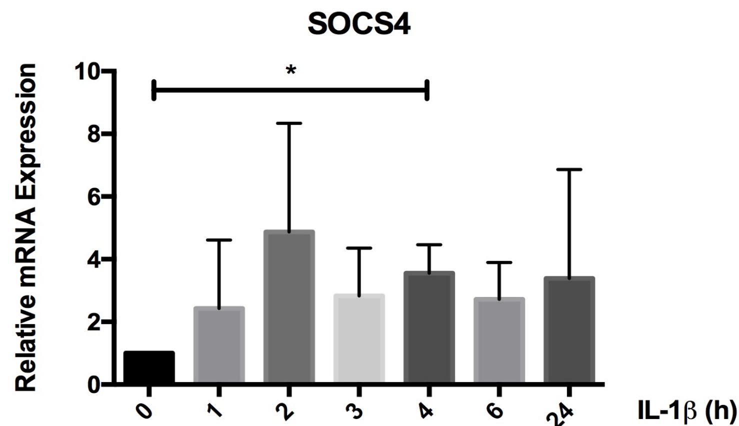

Figure 3.3. IL-1β induces SOCS4 mRNA level. HEK293T cells were stimulated with IL-1β (10ng/ml), after being rested for 2h in DMEM supplemented with 2% FBS. SOCS4 mRNA expression was normalised to the housekeeping gene, β-actin. All treated samples were displayed relative to the untreated (0) control, which was normalised to 1. The results are the mean (-/+ S.D) of three independent experiments and analysed by paired t-test *p<0.05.

After observing an upregulation of SOCS4 mRNA in response to IL-1β, we next investigated whether this upregulation translated to an increase in SOCS4 protein. HEK293T cells were treated with 10ng/ml of IL-1β for 0, 2, 4, 6 and 24h. The cells were then harvested and whole cell lysates were analysed by SDS-PAGE and blots probed with SOCS4 and β-actin antibodies. Figure 3.4A illustrates that SOCS4 protein was immediately induced in response to IL-1β treatment, but that its expression was most obvious after 24h. Indeed, while densitometric analysis **(Fig. 3.4B)**, of three independent experiments showed no statistical increase, there was a mean

increase of 1.4 at 2h, 1.3 at 4h, 1.6 at 6h and 4.2 at 24h compared to the untreated control.

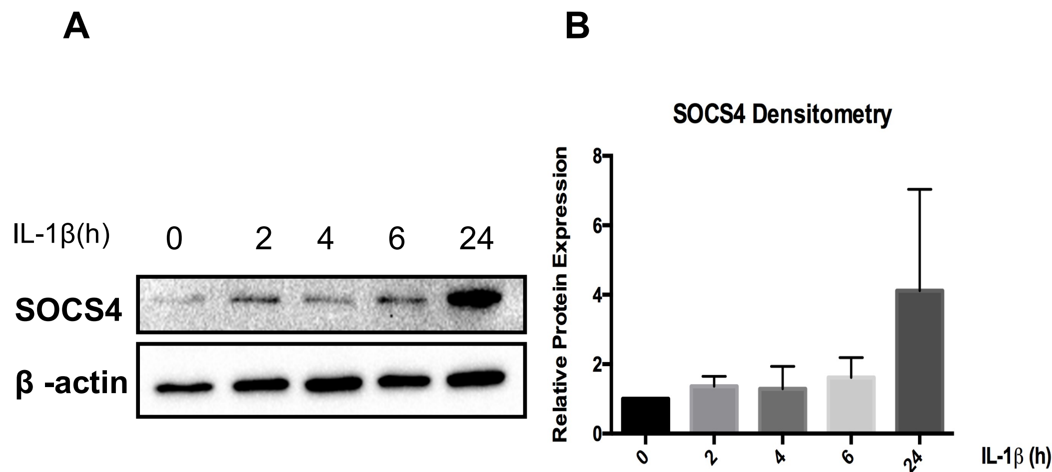

Figure 3.4: IL-1β treatment induces SOCS4 protein expression. (A) HEK293T cells were treated with IL-1β (10ng/ml), after being rested for 2h in DMEM supplemented with 2% FBS and lysates were analysed by SD-PAGE. Blots were probed with SOCS4 and β-actin antibodies. **(B)** Densitometric analysis of three independent experiments was carried out using Image lab software and the statistical analysis was performed by using prism. The bar graph illustrates the SOCS4 compared to the β-actin and IL-1β-treated sample values are displayed relative to the untreated (0) sample, which was normalised to 1.

3.2.3. TNF-α induces SOCS4 mRNA and protein.

Having observed that SOCS4 induction is regulated by IL-1β and IL-6, we wondered if that a third pro-inflammatory cytokine, TNF-α, also regulated SOCS4 expression. HEK293T cells were treated with TNF-α (20ng/ml) over a 24h time course. RNA was isolated and reverse transcribed to cDNA, which was used as template for qRT-PCR. The relative induction of SOCS4 mRNA was normalised to the β-actin house-keeping gene. Interestingly, while not statistically significant, there were subtle fluctuations in SOCS4 mRNA expression over the

first 6h of TNF-α treatment; however, there was a significant increase in SOCS4 mRNA (mean 2.3-fold increase) after 24h (p= 0.01) (**Fig.3.5)**.

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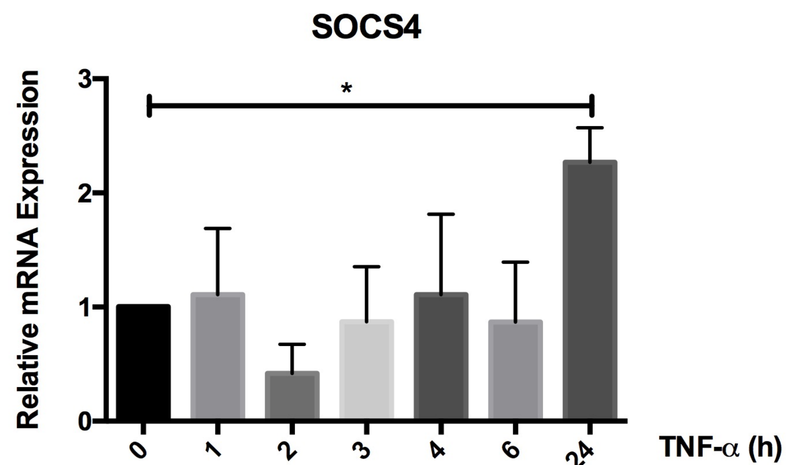

Figure 3.5: TNF-α induces SOCS4 mRNA expression. HEK293T cells were stimulated with TNF-α (20ng/ml), after being rested for 2h in DMEM supplemented with 2% FBS. SOCS4 gene expression was normalised to housekeeping gene, β-actin. All treated samples were displayed relative to the untreated (0) control, which was normalised to 1. Results are the mean (+/- SD) of three independent experiments and analysed by paired t-test *$p<0.05$

Having observed that TNF-α upregulates SOCS4 mRNA, we subsequently analysed the effect of TNF-α upon SOCS4 protein expression. HEK293T cells were treated over a 24h time course with 20ng/ml of TNF-α, before harvesting lysates and analysing them by Immunoblotting for SOCS4 and β-actin. The Western blots revealed that within 2h, TNF-α treatment increased SOCS4 levels and that similarly to the mRNA **(Fig. 3.5)**, levels of SOCS4 fluctuated in the first 6h (increased at 2h, dropped slightly at 4h and again increased at 6h). While the densitometric analysis did not identify any

statistically significant increases **(Fig. 3.6B)**, the mean SOCS4 levels increased 3.4-fold after 24h of TNF-α treatment, mirroring the significant mRNA induction **(Fig.3.5)**.

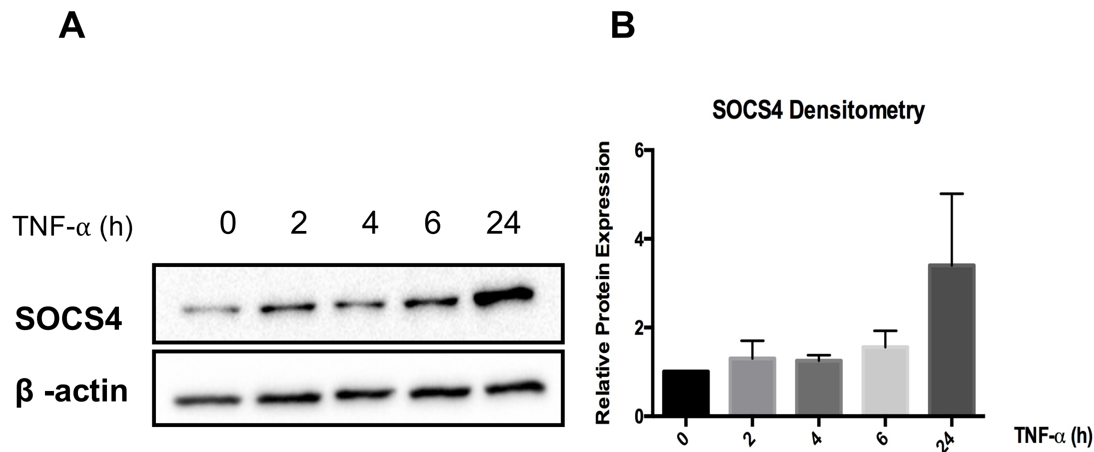

Figure 3.6: SOCS4 protein regulation upon TNF-α stimulation. (A) HEK293T cells were treated with TNF-α (20ng/ml), after being rested for 2h in DMEM supplemented with 2% FBS. Immunoblots were probed for SOCS4 and β-actin (loading control) antibodies **(B)** Densitometric analysis was carried out using Image Lab software and the statistical analysis was performed by using prism. Graph is the mean +/- SD of three independent experiments, SOCS4 compared to β-actin and TNF-α-treated sample values are displayed relative to the untreated (0) sample, which was normalised to 1.

3.2.4. IFN-α regulates SOCS4 mRNA induction.

Having determined that pro-inflammatory cytokines all induce SOCS4 expression, we next wondered if the key anti-viral Type 1 IFN, IFN-α, was also involved in SOCS4 regulation. To analyse this HEK293T cells were treated with IFN-α (1000IU/ml) over a time course (0, 1, 2, 3, 4, 6, 24h). We found that, while there was an immediate fold increase (1.8 mean fold increase at 1h), SOCS4 mRNA was

significantly increased (p=0.01) after 24h of IFN-α treatment **(Fig. 3.7)**.

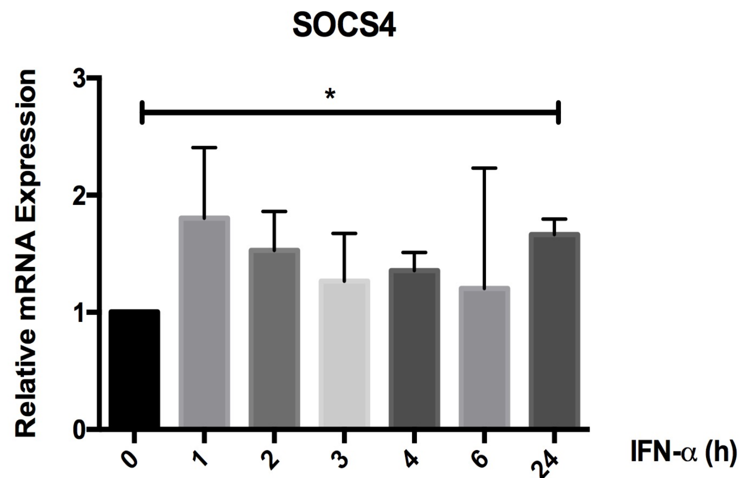

Figure 3.7: IFN-α induces SOCS4 mRNA induction. HEK293T cells were stimulated with IFN-α (1000IU/ml), after being rested for 2h in DMEM supplemented with 2% FBS. SOCS4 mRNA expression was normalised to the housekeeping gene, β-actin. All treated samples were displayed relative to the untreated (0) control, which was normalised to 1. The results are the mean (+/- S.D) of three independent experiments and analysed by Paired t-test *p<0.05

Having observed a significant induction at 24h, we next confirmed the expression of SOCS4 protein in response to IFN-α stimulation over a time course. HEK293T cells were treated with 1000IU/ml of IFN-α for 0, 2,4,6 and 24h, before harvested lysates were analysed by Immunoblotting for SOCS4 and β-actin. Western blot revealed that SOCS4 protein was upregulated over a time course until it reached the highest induction at 24h, which mirrors the mRNA induction pattern (**Fig 3.7)**. While the densitometric analysis did not identify any statistically significant increases **(Fig. 3.8B)**, the mean SOCS4 levels increased 1.3-fold after 24h.

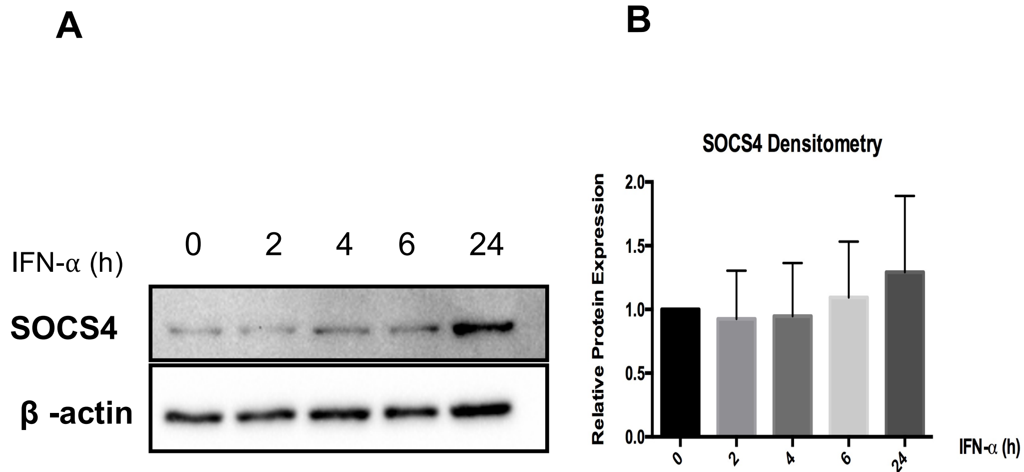

Figure 3.8: SOCS4 protein expressed in response to IFN-α stimulation. (A) HEK293T cells treated with IFN-α (1000 IU/ml) over a time course (0, 2, 4, 6 and 24h) after being rested for 2h in DMEM supplemented with 2% FBS.Immunoblots were probed for SOCS4 and β-actin (loading control) antibodies **(B)** Densitometric analysis was carried out using Image Lab software and the statistical analysis was performed by using prism. Graph is the mean +/- SD of three independent experiments, SOCS4 compared to the β-actin and IFN-α-treated sample values are displayed relative to the untreated (0) sample, which was normalised to 1.

3.3- The regulatory effect of SOCS4 upon JAK/STAT and NF-κB signaling:

Having confirmed that SOCS4 expression is induced by both pro-inflammatory and anti-viral cytokines (IL-6, IL1β, TNF-α and IFN-α). We investigated the effect of SOCS4 on intracellular signaling. As most SOCS proteins inhibit cytokine signaling through the JAK/STAT pathway [74], we first analysed the role of SOCS4 in JAK/STAT and NF-κB signal transduction. We transfected HEK293T cells with EV or SOCS4 plasmids for 24h. The cells were then “rested” for 2h in serum free media, before being stimulated with IL-6 (25ng/ml) and TNF-α

(20ng/ml), which signal via STAT3 and NF-κB pathway, respectively [131].

3.3.1- SOCS4 plasmid expression in HEK293T cells.

Initially, we transfected the SOCS4 (Flag-tagged) plasmid (or EV control) into HEK239T cells for 24h, before harvesting for total RNA and measuring SOCS4 mRNA levels by qRT-PCR. We observed a significant increase in SOCS4 mRNA (57-fold increase), compared to EV control (p =0.04) (**Fig. 3.9A**). HEK293T cells were also harvested for protein and SOCS4 protein levels were analysed by Immunoblotting using both a Flag or SOCS4 antibody. Constant protein levels were confirmed by blotting for β-actin. Indeed, we observed that the SOCS4 protein expression (**Fig. 3.9B and Fig. 3.9C**), mirrored that of the mRNA. In order to quantify the expression of SOCS4 protein expression upon transfection with the SOCS4 construct, immunoblots from Fig. 3.9C were analysed by densitometry. We found that while not statistically significant (p=0.21), SOCS4 protein certainly increased, when compared to the β-actin control **(Fig. 3.9D).**

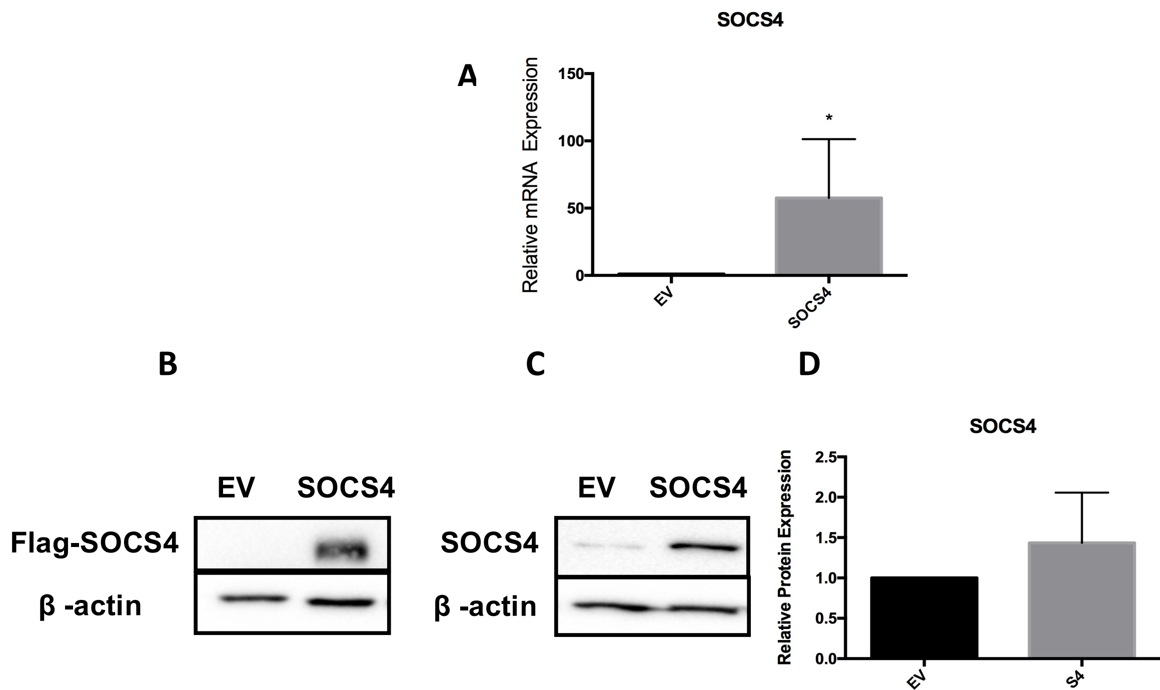

Figure 3.9: Confirmation of SOCS4 plasmid expression in HEK293T cells. HEK293T cells transfected for 24h with 2µg of construct encoding flag tagged SOCS4 or the EV control before **A)** cDNA was synthesised from total RNA and analysed by qRT-PCR for SOCS4 mRNA expression, which was normalised using the housekeeping gene, β-actin. SOCS4 levels were compared to cells transfected with EV control, which were normalised to 1. The graph shows the mean (+/- SD) of three independent experiments and analysed by using an unpaired Student's *t* test *p<0.05. HEK293T cells were also harvested for protein and cell lysates were analysed by western immunoblotting probing with **B)** Flag and β-actin antibodies (Blots are representative of two independent experiments) or **C)** SOCS4 and β-actin antibodies **D)** Densitometric analysis was carried out on immunoblots form Fig. 3.9C, SOCS4 protein levels were calculated as a ratio to β-actin, and SOCS4 transfected cells lysates were displayed as a comparison to EV transfected cells, which were normalised to 1. Graph is the mean +/- SD of three independent experiments.

3.3.2- SOCS4 reduces STAT3 phosphorylation.

Transfected cells were treated with IL-6 for 5min after being rested for 2h in serum free media. The cells were then harvested for protein lysates and analysed by immunoblotting. The western blot **(Fig. 3.10A)** shows a reduction in pSTAT3 in SOCS4 transfected cells treated with IL-6 when compared to treated EV. while not significant upon densitometry analysis **(Fig. 3.10B**) there was a mean fold decrease of pSTAT3 in SOCS4- transfected cells treated with IL-6 compared to EV transfected cells treated with IL-6. Indicating that SOCS4 has a role in controlling IL-6 signalling and regulates the JAK/STAT pathway.

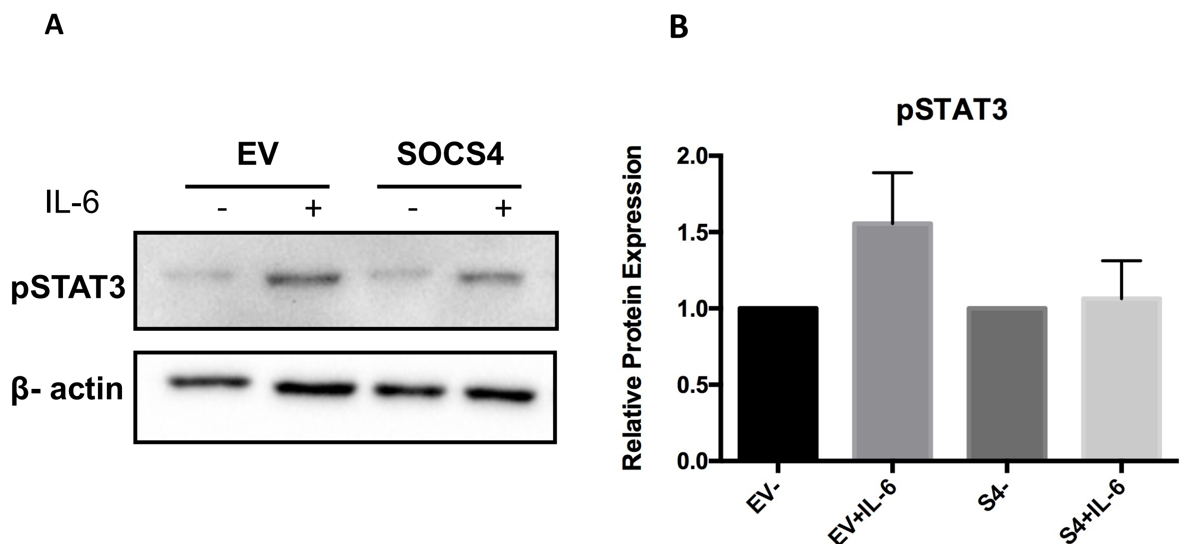

Figure 3.10. SOCS4 regulation of STAT3 phosphorylation after stimulation with IL-6. A) HEK293T cells were transfected with EV and SOCS4 (S4) plasmid constructs for 24h. Cells were rested for 2h in serum free media, then were stimulated with IL-6 (25ng/ml) for 5min. The membrane was probed for pSTAT3 and β-actin antibody**. B)** Densitometric analysis of three independent experiments was carried out using Image lab software and the statistical analysis

was performed by using prism. Bar graph is the mean +/- SD of three independent experiments and represents the ratio of pSTAT3 to β-actin band intensity relative to non-treated control which was normalised to 1.

3.3.3- SOCS4 does not inhibit TNF-α-mediated IκB-α degradation.

cells were transfected with the construct encoding SOCS4 or the EV control for 24h, and rested for 2h in serum free media, before treatment with TNF-α (20ng/ml) for 20min. The cells were harvested for protein lysates and analysed by immunoblotting. The western blot **(Fig.3.11A)** illustrates that stimulation with TNF-α induces the degradation of IκBα in the cells transfected with EV. In contrast, SOCS4 expression does not prevent TNF-α-mediated IκBα degradation. However, densitometry analysis **(Fig. 3.11B)** shows a reduction in IκBα degradation levels in the EV transfected cells treated with TNF-α compared to untreated EV, the reduction was close to significant (p=0.09). The level of IκBα degradation was significantly reduced in SOCS4 transfected cells treated with TNF-α when compared to untreated SOCS4 transfected cells (p= 0.04).

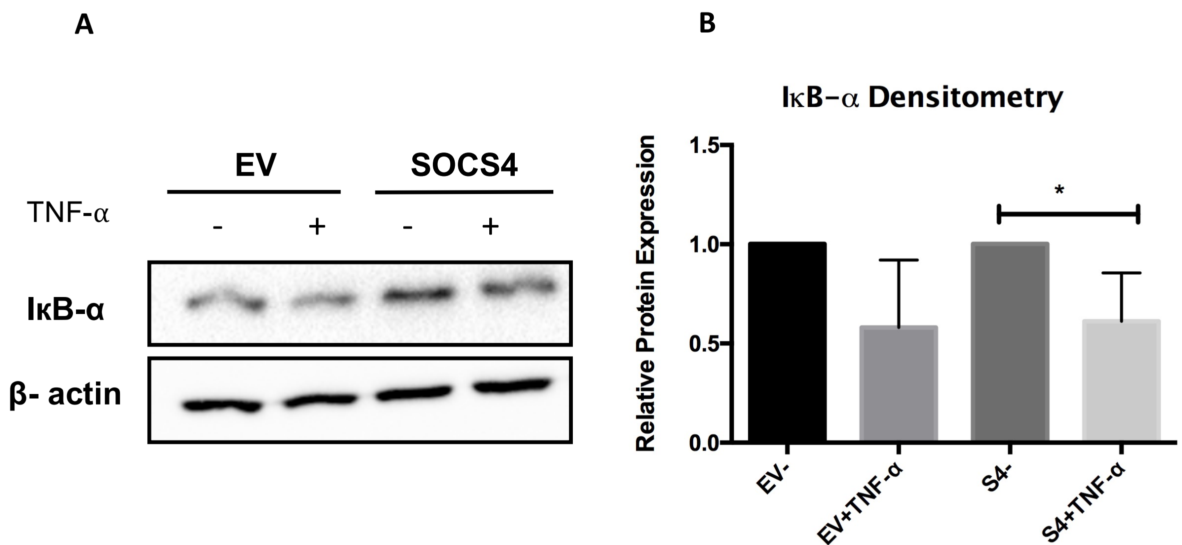

Figure 3.11. SOCS4 does not inhibit IκBα degradation after stimulation with TNF-α.

A) HEK293T cells were transfected with EV and SOCS4 plasmid constructs for 24h. Cells were rested for 2h in serum free media, and then were stimulated with TNF-α (20ng/ml) for 20min. Lysates were immunoblotted using IκBα and β-actin antibodies. Immunoblotting membranes were developed and analyzed using Bio-Rad gel Doc. **B)** Densitometric analysis of three independent experiments were done by using Image Lab and analysed by using an unpaired Student's *t* test using Prism software *p<0.05. Graph represents the ratio of IκBα to β-.actin band intensity relative to non-treated control which was normalised to 1 and the graph is the mean +/- SD of three independent experiments.

Chapter 4:

The cytokine-mediated regulation of SOCS5 and its role in IL-6 and TNF-α signalling

4.1- SOCS5 regulates pro-inflammatory cytokines and signal transduction.

Surprisingly, there remains little knowledge regarding the induction and regulatory effects of SOCS5, besides its key role in the negative regulation of EGF signalling. However, SOCS5-/- mice challenged with influenza, showed an increased viral load in the lung. In addition, SOCS5 directly inhibits JAK phosphorylation through its unique JAK inhibitory region (JIR). Therefore, given SOCS5's role in controlling influenza infection it is hypothesised that SOCS5 is involved in both the induction and signalling of pro-inflammatory and anti-viral cytokines. To investigate this hypothesis, we initially analysed the effect of key pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) and the antiviral cytokine, IFN-α, upon SOCS5 expression.

4.2- Investigating the effect of pro-inflammatory cytokines upon SOCS5 expression.

4.2.1. IL-6 regulation of SOCS5 mRNA and protein.

Since IL-6 regulates SOCS4 mRNA and protein expression, we investigated whether SOCS5 was also regulated in response to IL-6 treatment. In order to investigate this, HEK293T cells were stimulated with 25ng/ml of IL-6 over a 24h time course (0,1,2,3,4,6 and 24h), before harvesting for RNA, via the Phenol-chloroform method. SOCS5 mRNA levels were quantified relative to the housekeeping gene, β-actin, by qRT-PCR. While not significant, the data elucidates a trend of SOCS5 induction, most clearly at 3h and 6h, with mean fold induction (2.5 and 2.3 respectively). However, the induction of SOCS5 mRNA was close to significant after 4h ($p = 0.08$), indicating early induction of SOCS5 in response to IL-6 treatment **(Fig. 4.1).**

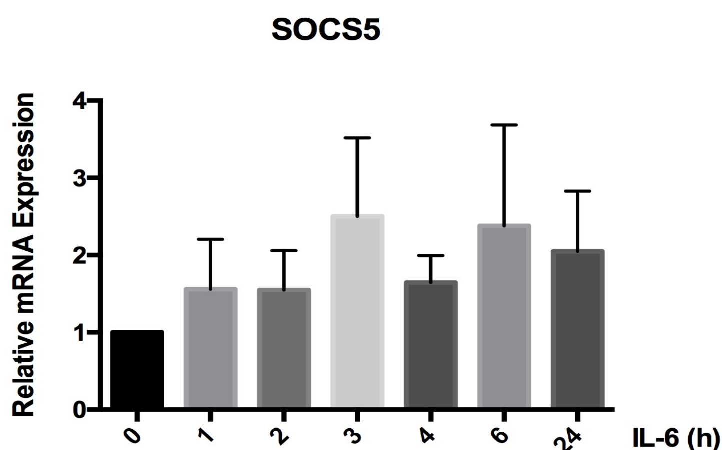

Figure 4.1: IL-6 regulation of SOCS5 mRNA. HEK293T cells were treated with IL-6 (25ng/ml) after being rested in DMEM supplemented with 2% FBS, for 0, 1, 2, 3, 4, 6 and 24h, before mRNA levels were measured by qRT-PCR. SOCS5 mRNA expression was normalised to the housekeeping gene, β-actin. IL-6 treated samples were displayed relative to the untreated "0", which was normalised to 1. The results are the mean (+/- SD) of three independent experiments.

Having observed an early increase in fold induction of SOCS5 mRNA in response to IL-6 treatment, next we sought to determine if this mRNA upregulation corresponded to an increase in SOCS5 protein. Therefore, HEK293T cells were treated with 25ng/ml of IL-6 for 0 , 2, 4, 6 and 24h, after being rested in DMEM supplemented with 2% FBS for 2h. The cells were then harvested and lysates were analysed by SDS-PAGE, before the Western blots were probed using SOCS5 and β-actin antibodies.

Figure 4.2A shows that SOCS5 protein was endogenously expressed at low levels (0h), but it began to increase after 2h of IL-6 treatment. Indeed, as we observed at the mRNA level **(Fig. 4.1)**, we observed SOCS5 protein to be highest after 24h. While not statistically

significant, densitometric analysis of three independent experiments revealed a continual increase in the amount of SOCS5 protein expressed over the 24h time course **(Fig. 4.2B)**.

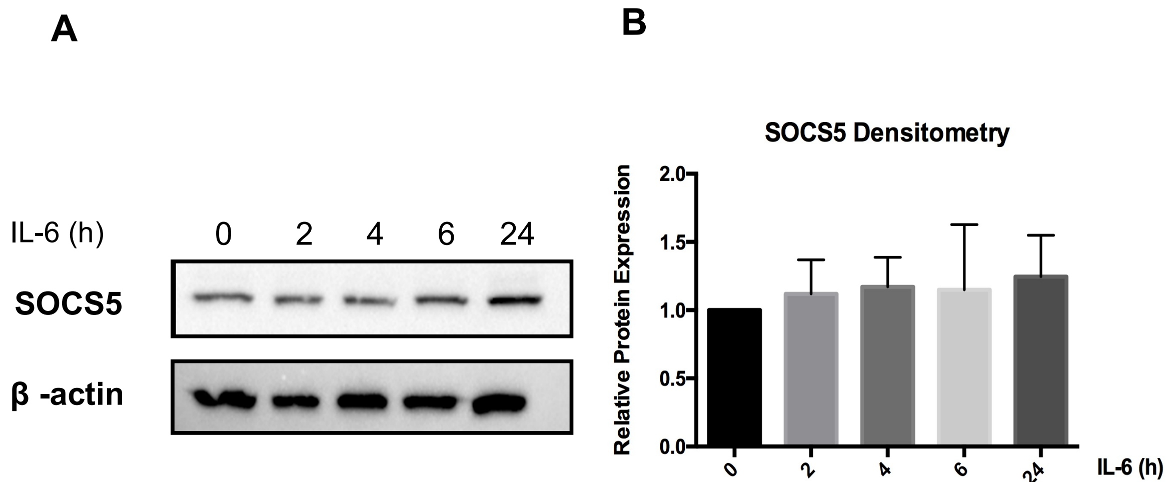

Figure 4.2: IL-6 induces SOCS5 protein. (A) HEK293T cells were treated with IL-6 (25ng/ml) for the indicated times, after being rested in DMEM supplemented with 2% FBS, and lysates were analysed by SDS-PAGE and Immunoblotting for SOCS5 and β-actin. Blots are representative of three independent experiments. **(B)** Densitometric analysis was performed using Image Lab software. Graph is the mean +/- SD of three independent experiments, SOCS5 compared to the β-actin and treated sample values are displayed relative to the untreated (0) sample which was normalised to 1.

4.2.2- IL-1β regulation of SOCS5 mRNA level and protein expression.

Having confirmed that IL-6 regulates SOCS5 mRNA, we were interested in analysing whether SOCS5 expression was also controlled by another key inflammatory cytokine, IL-1β. Firstly, we determined the regulation of SOCS5 mRNA levels, HEK293T cells were treated with IL-1β (10ng/ml) over a 24h time course(0, 1, 2, 3, 4, 6 and 24h). Cells were resuspended in TRI Reagent and the

isolated RNA was reverse transcribed to cDNA, which was used as template for qRT-PCR. mRNA levels of SOCS5 were quantified relative to the housekeeping gene, β-actin and the treated samples were compared to the untreated control, which was normalised to 1. The graph shows a trend of SOCS5 mRNA induction, but, as with IL-6, this is not statically significant. However, SOCS5 mean fold induction continued to increase over the IL-1β time course and reached 2.7-fold induction after 24h **(Fig. 4.3).**

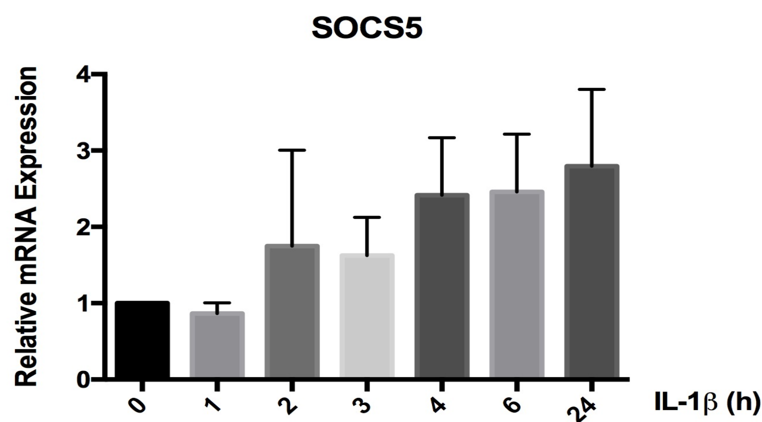

Figure 4.3: IL-1β regulation of SOCS5 mRNA induction. HEK293T cells treated with IL-1β (10ng/ml) for the indicated times after being rested in DMEM supplemented with 2% FBS for 2h. SOCS5 mRNA expression was normalised to the housekeeping gene, β-actin. All treated samples were displayed relative to the untreated (0) control, which was normalised to 1. The results are the mean (+/- S.D) of three independent experiments.

Next we examined the expression of SOCS5 protein in response to IL-1β stimulation. HEK293T cells were treated with 10ng/ml of IL-1β for 0, 2, 4, 6 and 24h. The cells were then harvested and cell lysates were analysed by SDS-PAGE and the blots probed with SOCS5 and β-actin antibodies. **Fig. 4.4A** shows significant induction of SOCS5 protein at 2h and 24h compared following IL-1β stimulation and

densitometry analysis shows a significant increase in protein expression at 2h when compared with non-treated cells (P = 0.04) **(Fig. 4.4B)**.

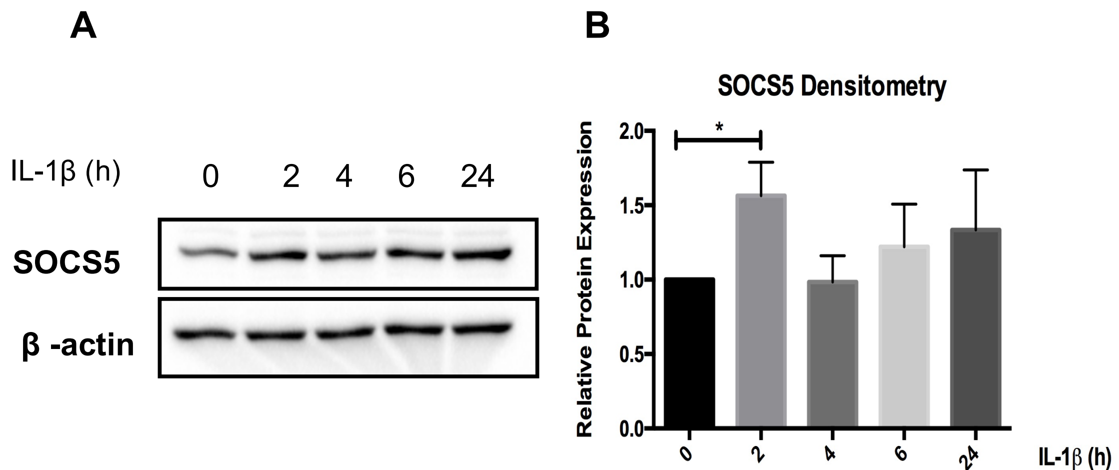

Figure 4.4: IL-1β induces SOCS5 protein expression. (A) HEK293T cells were rested in DMEM supplemented with 2% FBS, prior to stimulation with IL-1β (10ng/ml) over a 24h time course and lysates were analysed by SDS-PAGE. Blots were probed with SOCS5 and β-actin antibodies. **(B)** Densitometric analysis of three independent experiments was carried out using Image Lab and analysed statistical analysis was performed using Prism. Graph is the mean +/- SD of three independent experiments. Levels of SOCS5 were compared to the β-actin and displayed relative to the untreated (0) control, which was normalised to 1. *p<0.05 (paired t-test).

4.2.3- TNF-α regulation of SOCS5 mRNA and protein.

Having observed that SOCS5 levels were regulated by IL-1β and IL-6, we wondered if a third proinflammatory cytokine, TNF-α, also regulated SOCS5 expression. HEK293T cells were treated with 20ng/ml of TNF-α over a 24h time course. RNA was isolated and reverse transcribed to cDNA, which was used as template for qRT-

PCR. The relative induction of SOCS5 mRNA was normalised to the β-actin house-keeping gene. While not statistically significant, an early upregulation of SOCS5 expression after 2h continued to 6h. However, unlike IL-6 and IL-1 β, SOCS5 was not induced after 24h stimulation **(Fig. 4.5)**.

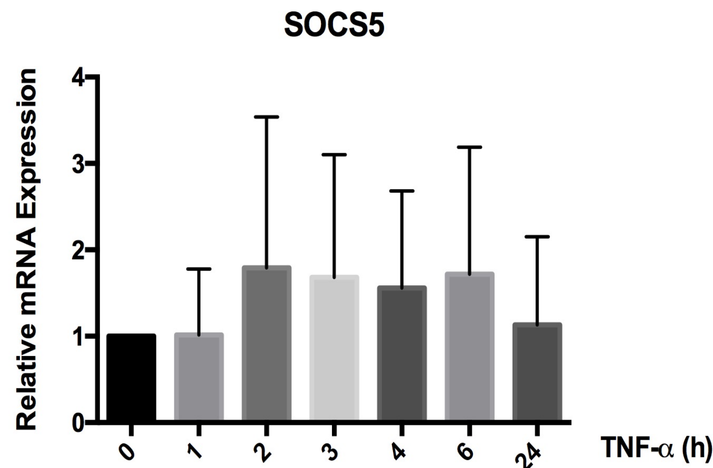

Figure 4.5: TNF-α regulation of SOCS5 mRNA induction. HEK293T cells were stimulated with TNF-α (20ng/ml) after being rested in DMEM supplemented with 2% FBS. SOCS5 gene expression was normalised to housekeeping gene, β-actin. All treated samples were displayed relative to the untreated (0) control, which was normalised to 1. Results are the mean (-/+ SD) of three independent experiments

Having observed not statistically significant effect in SOCS5 mRNA upon TNF-α treatment, we next analysed its effect upon SOCS5 protein expression. HEK293T cells were treated over a 24h time course with 20ng/ml of TNF-α before lysates were generated and analysed by immunoblotting for SOCS5 and β-actin antibodies. The western blots **(Fig. 4.6A)** revealed that TNF-α treatment subtly increased SOCS5 protein levels after 24h. Interestingly, while we had

not observed the same SOCS5 mRNA increase at 24h **(Fig. 4.5)** it is possible that the continual earlier increase (observed after 2-6h) acted to increase protein levels by 24h. Densitometry analysis did not identify any statistically significant increases (**Fig 4.6B)**, but the mean SOCS5 levels increased after 24h, did suggest a defined upregulation of SOCS5 protein upon TNF-α treatment.

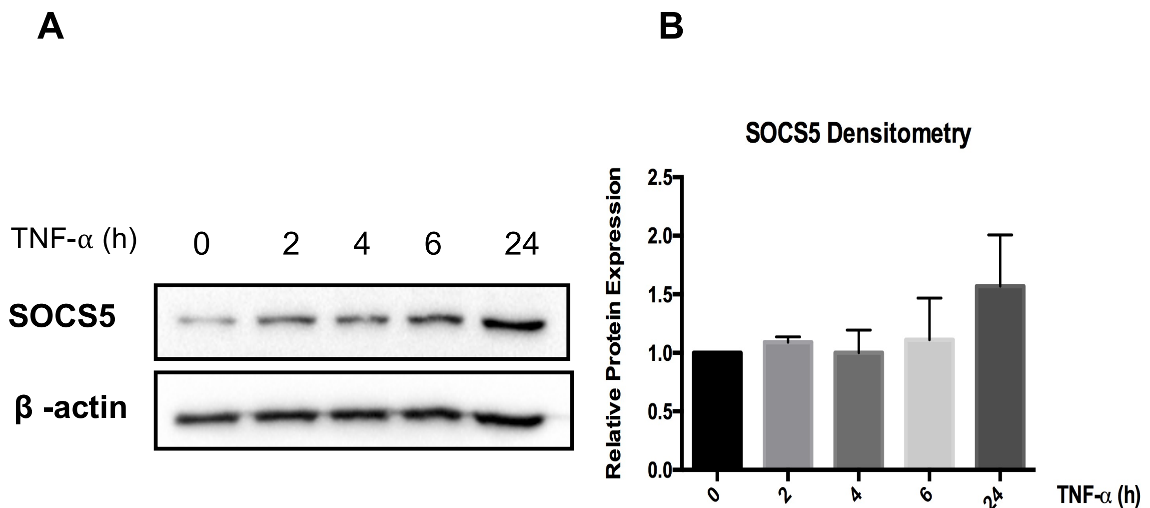

Figure 4.6: SOCS5 protein levels in response to TNF-α stimulation.

(A) HEK293T cells were treated with TNF-α (20ng/ml) for the indicated times after being rested in DMEM supplemented with 2% FBS for 2h, and lysates were analysed by SDS-PAGE. Blots were probed with SOCS5 and β-actin antibodies. **(B)** Densitometric analysis of three independent experiments was carried out using Image Lab. Graph is the mean +/- SD of three independent experiments. Levels of SOCS5 were compared to the β-actin and displayed relative to the untreated (0) sample, which was normalised to 1.

4.2.4- IFN-α regulation of SOCS5 mRNA and protein.

Having observed SOCS4 upregulation in response to IFN-α (significantly upregulated after IFN-α stimulation for 24h **(Fig. 3.7)**), we were interested to determine if IFN-α also plays a role in SOCS5

expression. HEK293T cells were treated with IFN-α (1000IU/ml) over a time course (0, 1, 2, 3, 4, 6, 24h). We found that a trend of SOCS5 mRNA increased in response to IFN-α stimulation and reached 2-fold increase at 2h. However, while the mean SOCS5 levels decreased after 6h, they appeared to slightly increase again after 24h **(Fig. 4.7)**.

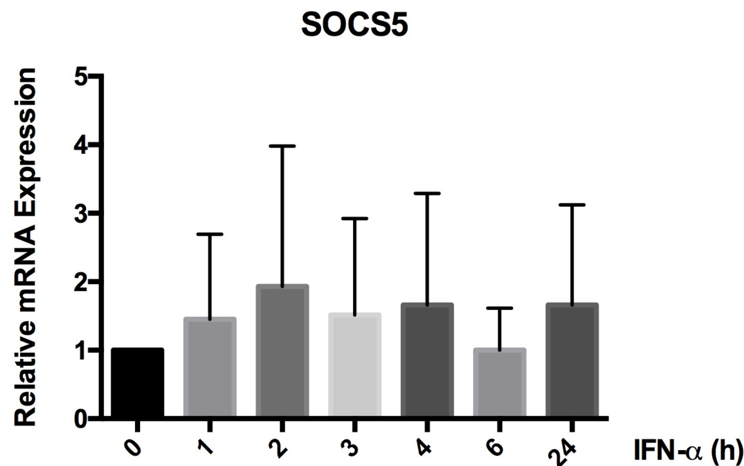

Figure 4.7: IFN-α regulation of SOCS5 mRNA induction. HEK293T cells were stimulated with 1000IU/ml IFN-α for the indicated times after being rested in DMEM supplemented with 2% FBS for 2h. The cells were harvested and the isolated RNA was reverse transcribed to cDNA and used as a template for qRT-PCR. mRNA expression was normalised to the housekeeping gene, β-actin and normalised to the non-treated control. Results are the mean (+/- S.D) of three independent experiments.

Having observed an increase in mean levels of SOCS5 mRNA after IFN-α treatment, we next analysed the effect of IFN-α upon SOCS5 protein levels. HEK293T cells were treated with 1000IU/ml of IFNα for 0, 2, 4, 6 and 24h, before lysates were harvested and analysed by immunoblotting for SOCS5 and β-actin. The western blot (**Fig 4.8A**), revealed that IFN-α treatment subtly increased SOCS5 levels at 2, 4

and 24h while there was a small decrease at 6h, which all mirrored the mRNA induction pattern (**Fig 4.7)**. Based on dosimetry analysis, it appears there is a slight increase in SOCS5 **(Fig. 4.8B).**

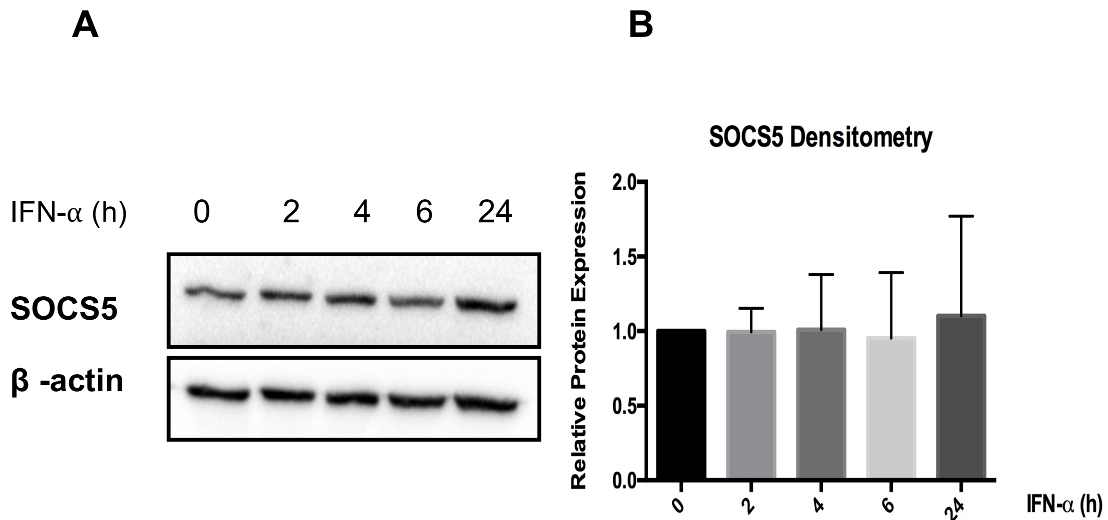

Figure 4.8: SOCS5 protein regulation in response to IFN-α stimulation. (A) HEK293T cells were treated with 1000IU/ml IFN-α over a 24h time course and lysates were analysed by SDS-PAGE. Blots were probed with SOCS5 and β-actin antibodies. **(B)** Densitometric analysis of three independent experiments was carried out using Image Lab software. The graph is the mean +/- SD of three independent experiments. Levels of SOCS5 were compared to the β-actin and displayed relative to the untreated (0) sample, which was normalised to 1.

4.3- The regulatory effect of SOCS5 upon JAK/STAT and NF-κB signalling:

Having shown previously that SOCS4 plays a role in regulating signalling pathways in particular the JAK/STAT and NF-κB pathways. We were keen to investigate the role of SOCS5 in these signalling pathways. HEK293T cells were seeded at a density of 2.5×10^5 cell/ml in 6-well plates. The cells were transfected with EV and SOCS5 plasmid constructs at 2μg/well for 24h. Before treatment with the

cytokines of interest, the cells were rested in serum free media (SFM) for 2h.

4.3.1- Confirmation of the expression of SOCS5 plasmid construct:

A construct encoding SOCS5 (Flag-tagged) or the EV control were transfected into HEK293T cells for 24h. Post-transfection, cells were harvested for RNA which was then reverse transcribed into cDNA. SOCS5 mRNA levels were then analysed by qRT-PCR. We observed a significant induction in SOCS5 mRNA (2.1-fold), compared to EV (P= 0.03) (**Fig. 4.9A**). Transfected HEK293T cells were also harvested for protein and SOCS5 protein levels were analysed by immunoblotting using a SOCS5 antibody. Constant protein levels were confirmed by blotting for β-actin. Indeed, we observed that the SOCS5 protein expression (**Fig. 4.9B and Fig. 4.9C**), mirrored that of the mRNA. In order to quantify the expression of SOCS5 protein expression upon transfection with the SOCS5 construct, immunoblots from Fig. 4.9C were analysed by densitometry. We found that, while not statistically significant (p=0.09), SOCS5 protein increased, when compared to the β-actin control.

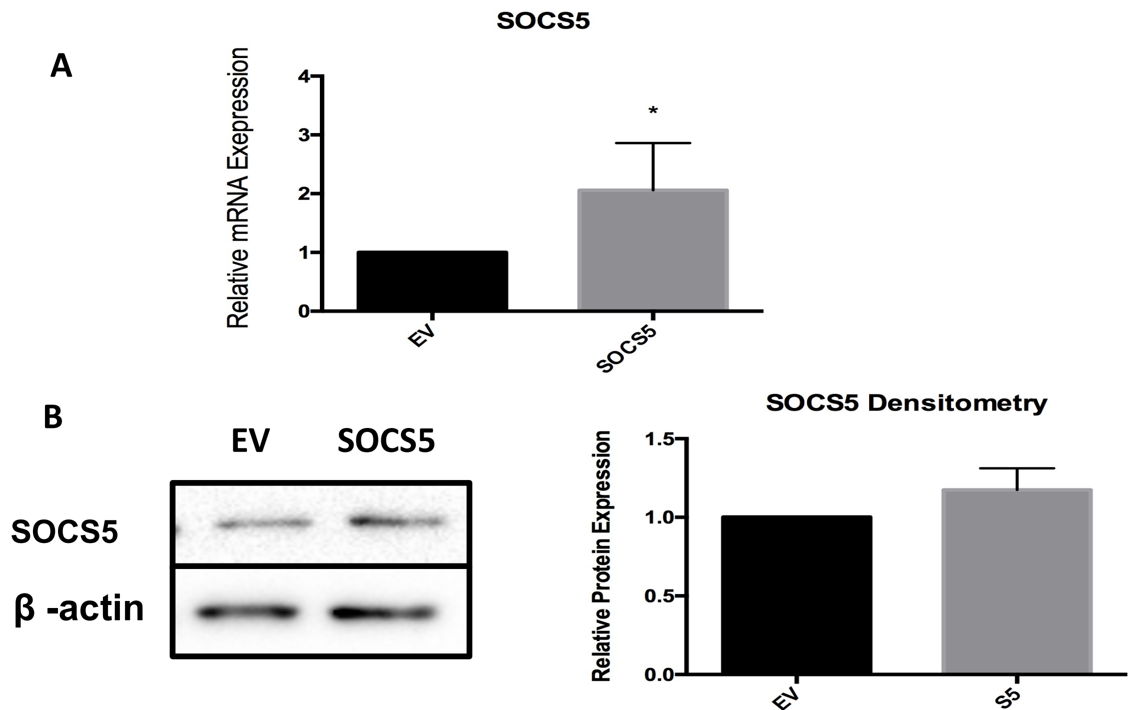

Figure 4.9. Confirmation of SOCS5 expression in HEK293T cells. HEK293T cells were transfected with 2µg of EV or a SOCS5 encoding plasmid for 24h . **A)** cDNA was synthesised from extracted RNA and analysed by qRT-PCR for SOCS5 mRNA expression, which was normalised using the housekeeping gene, β-actin. SOCS5 transfected cells were compared to cells transfected with EV control, which were normalised to 1. The graph shows the mean (+/- SD) of three independent experiments and analysed by using an unpaired Student's *t* test, *$p<0.05$ **B)** HEK293T cells were harvested for protein and cell lysates were immunoblotted for anti-SOCS5 antibodies (Blot is representative of three independent experiments). All blots were probed for β-actin to confirm equal loading. **C)** Densitometric analysis compared SOCS5 protein levels were calculated as a ratio to β-actin and displayed relative to EV transfected cells, which were normalised to 1. Graph is the mean +/- SD of three independent experiments.

4.3.2- SOCS5 enhances IL-6 mediated STAT3 phosphorylation.

Having observed an mean fold induction of SOCS5 upon IL-6 treatment we wondered if SOCS5 expression acted to regulate IL-6

signalling. Therefore, to analyse this SOCS5 transfected cells were treated with IL-6 (25ng/ml) for 5min after being rested in SFM for 2h. The cells were then harvested for protein, and the lysates were analysed by SDS-PAGE. The western blot revealed that the normal IL-6-induced pSTAT3 levels (lane 3) were enhanced upon expression of SOCS5. Furthermore, SOCS5 expression enhances pSTAT3 levels compared to EV transfected cells upon IL-6 treatment **(Fig. 4.10A)**. In addition to the observed visual increase in pSTAT3, enhanced phosphorylation was confirmed by densitometric analysis in SOCS5 transfected cells treated with IL-6, compared to EV **(Fig. 4.10B)**. We had hypothesised that SOCS5 would suppress IL-6 signalling. However, surprisingly, these findings suggest that SOCS5 has a role in the positive regulation of the pro-inflammatory IL-6 pathway.

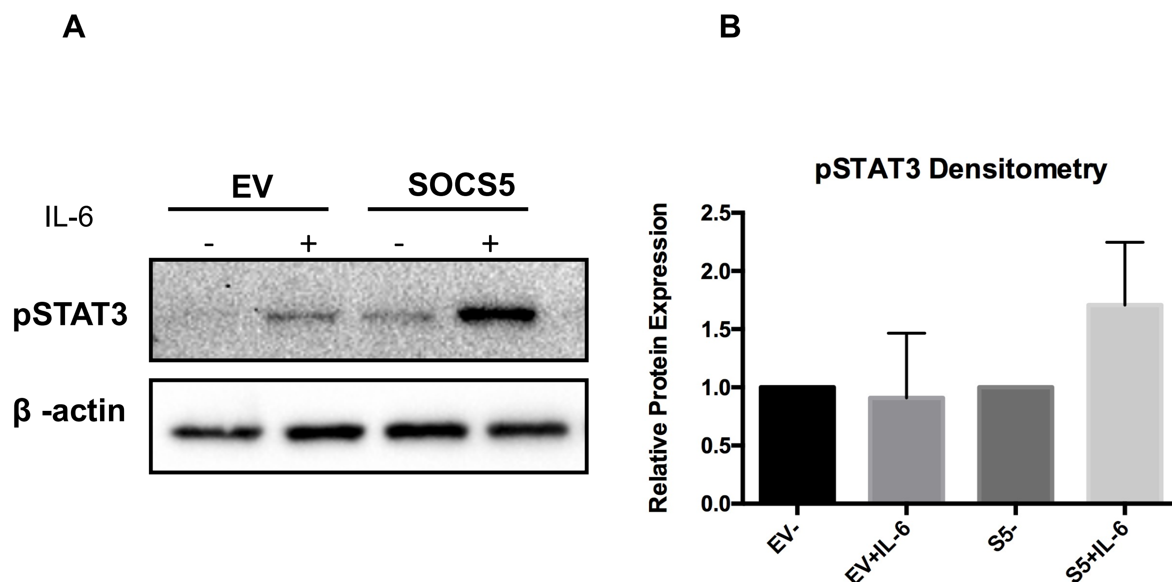

Figure 4.10- SOCS5 enhances pSTAT3 after IL-6 treatment.

A) HEK293T cells were transfected with EV and SOCS5 encoding constructs. 24h post-transfection, cells were rested in SFM for 2h and then stimulated with IL-6 (25ng/ml) for 5 minutes. Lysates were analysed by SDS-PAGE and the membranes were were immunoblotted with pSTAT3 and β-actin antibodies. **B)** Densitometric analysis was done using Image Lab software. The graph

represents the ratio of pSTAT3 to β-actin band intensity relative to non-treated control which was normalised to 1. The graph is the mean +/- SD of three independent experiments.

4.3.3- SOCS5 inhibits TNF-α-mediated IκB-α degradation.

SOCS5 transfected cells were treated with TNF-α (20ng/ml) for 20min after being rested for 2h in SFM. Western immunoblotting revealed IκB-α degradation following stimulation with TNF-α, was reduced in the presence of SOCS5. **(Fig. 4.11A).** Indeed, the densitometry analysis of three independent experiments revealed that the significant reduction (p=0.008) in IκBα protein upon treatment of TNF-α observed in EV-transfected cells, was lost in cells transfected with SOCS5. Therefore, these results indicate that SOCS5 inhibits TNF-α-mediated IκB-α degradation.

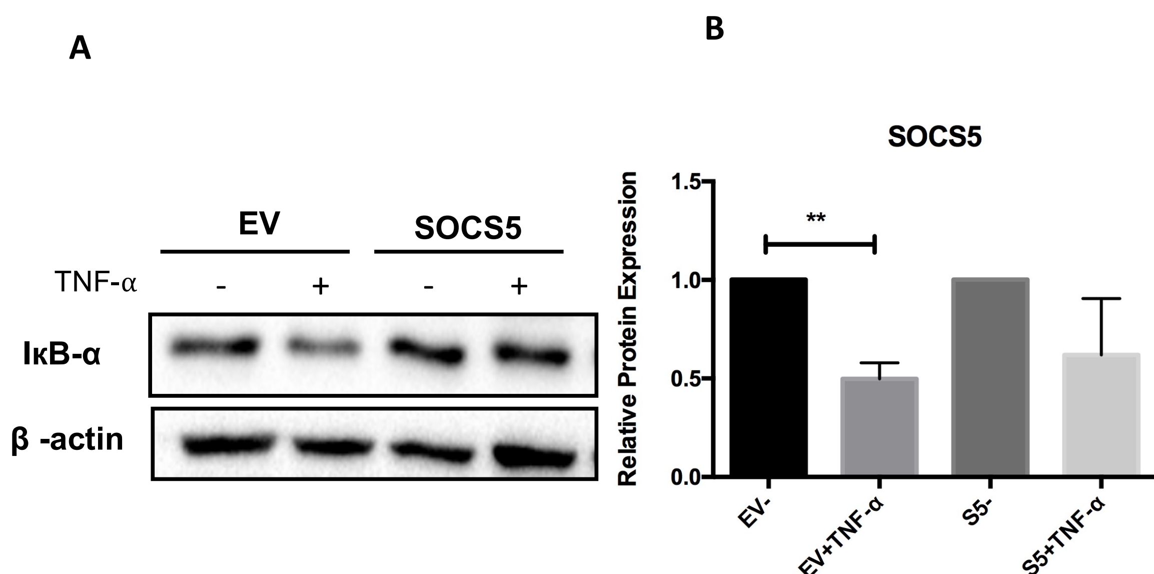

Figure 4.11- SOCS5 inhibits TNF-α-mediated IκB-α degradation.

A) HEK293T cells were transfected with EV and SOCS5 plasmid constructs for 24h. Post transfection, cells were rested in SFM for 2h and stimulated with TNF-α (20ng/ml) for 20min. Lysates were immunoblotted using IκBα and β-actin antibodies. Blots were analyzed using Bio-Rad gel Doc. **B)**

Densitometric analysis of three independent experiments were done using Image Lab software and analysed by Student's *t* test using Prism software, $^{**}p<0.01$. Bar graph represents the ratio of IκBα to β-actin band intensity relative to non-treated controls which were normalised to 1.

Chapter 5
Discussion

Since the discovery of SOCS proteins, many studies have confirmed their main function as inhibitors of cytokines-induced signalling. They classically regulate signalling via inhibition of JAK-associated cytokine receptor complexes, which effectively leads to the control of both the innate and adaptive immune responses [74]. Much of our understanding regarding SOCS function originates from studies involving SOCS1-3 and CIS [74]. Despite the development in this area, the function of SOCS4-7 is still poorly defined.

The physiological roles of SOCS4 and SOCS5 have been examined in a limited number of studies, however, while these investigations provide a "starting point", our understanding of these two SOCS family members remains poor. To date, SOCS4 and SOCS5 knockout mice have been used to investigate their role in inhibiting specific cytokine signaling pathways. Using a virulent influenza infection model, SOCS4-deficient mice were found to resist infection and exhibited an increase in the expression level of pro-inflammatory cytokines, such as IL-6 and IL-1β [111]. Moreover, this study also showed a delay in viral clearance, due to impaired trafficking of virus-specific CD8+ T cells to the site of infection [111]. Additionally, SOCS4 has been found to block EGF signalling via EGFR proteasomal degradation [109]. SOCS5 is the closest related protein to SOCS4, as they share 92% sequence identity of their SH2 domain [108]. Despite this, it is still unclear if their biological roles correspond to their sequence homology [83]. It has been found that SOCS5 can interact independently via its N-terminal conserved interaction region (JIR), which facilitates direct binding to JAK1 and JAK2. This region

also mediates the interaction of SOCS5 with both the EGF and IL-4 receptors [132].

Therefore, since there is a lack of basic knowledge regarding SOCS4 and SOCS5, this project aimed to investigate the effect of a spectrum of cytokines upon SOCS4 and SOCS5 expression and the role of these SOCS in controlling pro-inflammatory cytokine signalling.

5.1- The role of SOCS4 in IL-6 signalling

Since IL-6 was the most highly elevated pro-inflammatory cytokine in SOCS4-deficient mice [111], we sought to investigate the role of SOCS4 in IL-6 signalling. We firstly analysed if IL-6 enhanced the expression of SOCS4. We treated HEK293T cells with IL-6 over a time course and found that SOCS4 mRNA was induced as early as 2h, with induction significantly doubling after 24h. Since SOCS4 was induced by IL-6 we hypothesised that it might also be acting to negatively regulate IL-6 signalling. SOCS3 acts in a negative feedback loop to limit IL-6 signalling by binding with SHP-2 (a positive regulator to Tyr-757 on gp130) [80]. Therefore, to determine if SOCS4 also plays a role in inhibiting IL-6 JAK/STAT signalling, we overexpressed a SOCS4 plasmid, before treating it with IL-6. We found that as previously observed with SOCS3, IL-6-mediated STAT3 phosphorylation was also suppressed by SOCS4. While this an exciting discovery, it paves the way for future studies to analyse the exact mechanism by which SOCS4 might inhibit pSTAT3. Since SOCS4 has been reported to be upregulated upon EGFR phosphorylation, (by binding to Tyr-1092) and thus reduces STAT3

signalling from EGFR through receptor degradation. Therefore, future experiments may reveal that SOCS4 downregulates IL-6 signalling by a similar mechanism.

5.2- The regulation of SOCS4 by IL-1β

Since SOCS4-deficient mice also expressed high levels of IL-1β [111] and given that SOCS1 has been shown to negatively regulate IL-1β signalling at several levels, (via c-Jun N-terminal kinase (JNK), p38 and nuclear NF-κB) [133], we were interested in studying the effect of IL-1β upon SOCS4 induction. We found that cells treated with IL-1β had increased levels of SOCS4 mRNA as early as 2h, which rose significantly at 4h. While the induction of SOCS4 mRNA by IL-1β reached significance earlier than IL-6-induced SOCS4 mRNA, it is clear that both cytokines upregulate SOCS4. We also observed SOCS4 protein induction following 2h and 6h stimulation with IL-1β, which was further enhanced following 24h treatment. While this gradual increase in SOCS4 does not match the more transient mRNA observation, it is possible that the earlier induction of SOCS4 mRNA by IL-1β led to the continual increase in SOCS4 protein. In future studies, extension of our time course, beyond 24h is likely to reveal a more comprehensive understanding of IL-1β induction of SOCS4 protein.

5.3- The interplay between SOCS4 and TNF-α signalling.

Our lab has previously published that HCV induces SOCS3 [106]. Indeed, my colleagues were the first to discover that SOCS3 directly inhibits TNF-α-induced NF-κB activation [106]. These findings showed that SOCS3 protein can regulate both JAK/STAT and TNF-α

signalling pathways [134]. Therefore, these revelations led us to investigate the effect of TNF-α on SOCS4 expression. HEK293T cells were treated with TNF-α and we found that SOCS4 mRNA induction fluctuated over a 24h time course. However, SOCS4 mRNA and protein were significantly induced after 24h.

TNF-α binds to the TNFR1 receptor and triggers signalling via activation of IκBα and translocation of NF-κB into the nucleus [135]. Having previously published that SOCS3 controls TNF-α-mediated NF-κB signalling and having now observed an induction of SOCS4 by TNF-α, we wondered if SOCS4 could also regulate TNF-α signalling. To investigate this, we transfected cells with a SOCS4 construct and treated them with TNF-α for 20min before analysing IκBα. We found that overexpression of SOCS4 did not inhibit IκBα degradation, compared to EV control cells.

These findings reveal that while TNF-α induces expression of SOCS4, SOCS4 does not inhibit TNF-α-mediated NF-κB signalling. Our findings were similar to that of IL-10-induced SOCS3, which SOCS3 does not inhibit IL-10 signalling, but instead SOCS3 controls IL-6 signalling [136]. Therefore, future studies should investigate if TNF-α induced SOCS4 regulates other signaling pathways.

5.4- The role of IFN- α in SOCS4 and SOCS5 expression

Type 1 IFNs are produced in response to viral infection. Compared to WT mice, SOCS4-knockouts challenged with influenza virus demonstrated a delay in viral clearance, highlighting the role of SOCS4 in controlling anti-viral responses [111]. Therefore, we hypothesised that SOCS4 could be induced by the Type 1 IFN, IFN-α. To test this hypothesis, we treated HEK293T cells with IFN-α and

found an up-regulation of SOCS4 mRNA, which was statistically significant after 24h. Furthermore, this translated to the protein level with induction SOCS4 protein highly increased at 24h.
Although the JAK/STAT signalling pathway is the major modulator of Type I IFN signalling, IFN-α also induce phosphorylation of ERK, a key step in the STAT-independent MAPK pathway [137], revealing how IFN-α regulates and activates different pathways to exert its effects upon a given cell. Therefore, future studies analysing the role of IFN-α-induced SOCS4 should analyse its effect upon both JAK/STAT and MAPK signaling pathways.
In contrast, even though SOCS5-deficient mice show an elevated pro-inflammatory response, type I IFN levels are decreased at day one post-influenza infection, revealing a loss in normal anti-viral activity [130]. To further elucidate the role of SOCS5 during anti-viral immunity, we treated HEK293T cells with IFN-α and analysed SOCS5 levels. SOCS5 mRNA and protein showed “slight” increase, which dropped after 6h and increased again at 24h. Since SOCS5-/- mice have enhanced viral replication, indicating that SOCS5 mediated a normal anti-viral response, it was not surprising that we found upon IFN-α stimulation, SOCS5 was induced. However, future studies should also analyse the role of SOCS5 in regulating Type I IFN signalling.

5.5- The role of SOCS5 in IL-6 signalling

SOCS5 has been shown to interact with JAK proteins, the EGFR and IL-4R [132]. Studies on SOCS protein function have found that SOCS3 and SOCS1 inhibit IL-6 signalling [138]. In line with these

studies, we hypothesised that IL-6 might induce SOCS5. We found that IL-6 indeed upregulates SOCS5 mRNA and while not statistically significant, there was an increase in SOCS5 protein expression which was strongest at 24h. Having observed an increase in SOCS5 upon IL-6 treatment, we then proceeded to investigate whether SOCS5 acts to negatively regulate IL-6 signalling by examining STAT3 phosphorylation. We transfected cells with a plasmid encoding SOCS5 or a control (empty vector), and then treated with IL-6. To our surprise, we found that overexpression of SOCS5 enhanced IL-6-induced STAT3 phosphorylation. Our results cast a new light on the biological role of SOCS5 in this signalling pathway. SOCS3 has been reported to inhibit pSTAT3 in response to IL-6 stimulation, via binding to receptor phospho-tyrosine residues and blocking STAT3 recruitment to receptor [138]. Studies on SOCS2 showed that it can interfere with the negative effect of SOCS3, revealing direct cross-modulation between SOCS proteins. Also, SOCS2 has also been reported to have dual effects on growth hormone (GH) signal transduction. While SOCS1 and SOCS3 classically inhibited the transcriptional activation of the GH-responsive element and suppressed JAK2 tyrosine kinase activity, SOCS2 had two opposite effects: at low concentrations SOCS2 inhibited GH-induced STAT5-dependant gene transcription and at high concentrations it restored GH signalling. SOCS2 also enhances IL-2 and IL-3-induced STAT phosphorylation in response to cytokine stimulation via proteasome-dependent reduction of SOCS3 and SOCS1 protein expression[100, 101, 139]. Therefore, it is possible that SOCS5 functions in a similar way to SOCS2 and might suppress another regulator (e.g. SOCS3), which would then allow pSTAT3 to be enhanced in the presence of IL-6. This novel data indicates that SOCS5 may not only attenuate

cytokine signalling, but it may also contribute to its enhancement, thus revealing SOCS5 as an intricate “conductor” of biological responses.

5.6- The role of IL-1β in SOCS5 induction

Having found that SOCS5 was induced by IL-6, we next investigated if a second pro-inflammatory cytokine could induce SOCS5. Thus, we measured mRNA and protein expression of SOCS5 in response to IL-1β. Upon stimulation with IL-1β, SOCS5 mRNA levels immediately began to increase, which continued even up to 24h. Interestingly, while SOCS5 protein expression shows a similar pattern of continual increase, its induction was statistically significant after 2h ($p=0.04$), compared to untreated controls. Our data revealed for the first time that SOCS5 mRNA and protein expression are increased in response to IL-1β stimulation, although further investigations are required to determine a role for SOCS5 in the regulation of IL-1β signalling.

5.7- The role of SOCS5 in TNF-α signalling

Studies of SOCS5 have focused on pro-inflammatory cytokines [130]. However, we have found that SOCS4 is induced in response to TNF-α, without inhibiting TNF-α-mediated NF-κB activation. Conversely, SOCS3 is known to be induced by TNF-α and inhibit its NF-κB signal transduction [106]. Hence, we were interested to investigate the induction of SOCS5 in response to TNF-α stimulation and determine if SOCS5 regulated TNF-α signalling.

SOCS5 mRNA and protein was increased throughout the time course of treatment with TNF-α. TNF-α is known to promote signal transduction and the activation of NF-κB, which requires IκBα degradation and translocation of NF-κB into the nucleus [135]. HEK293T cells were transfected with a construct encoding SOCS5. The role of SOCS5 in TNF-α-mediated NF-κB activation was subsequently analysed by measuring the degradation of IκBα. Our findings revealed less IκBα degradation in SOCS5-expressing cells, compared to cells transfected with the EV control, upon TNF-α stimulation. This indicates that SOCS5 inhibits TNF-α-mediated IκBα degradation. As this has not been investigated before, our findings have highlighted a novel biological role of SOCS5 and most importantly we have identified a new regulator of TNF-α signalling. Since TNF-α is involved in a variety of inflammatory conditions, including rheumatoid arthritis and inflammatory bowel diseases [20-22], our results have revealed a new regulator (SOCS5) of this key cytokine, and this may be useful in our quest to therapeutically control autoimmunity.

Conclusion and Future

Overall, our findings have shown for the first time that pro-inflammatory and anti-viral cytokines induce both SOCS4 and SOCS5. Furthermore, we have shown that even though TNF-α induces both SOCS4 and SOCS5, only SOCS5 inhibits TNF-α-mediated NF-κB signal transduction. Indeed, our results reveal that SOCS4 and SOCS5 also have differential effects upon IL-6-mediated STAT3 signalling; SOCS4 suppresses its phosphorylation, while SOCS5 enhances it. Future studies should investigate if SOCS5, acts like SOCS2, to inhibit other SOCS (e.g. endogenous SOCS3), which leads to the observed increase in STAT3 activation. Indeed, our results indicate that SOCS5 might have a similar "dual effects" like SOCS2, and function as both an enhancer and suppressor of cytokines signalling.

These findings show both SOCS4 and SOCS5 to have previously undescribed roles in controlling immune responses that will form the basis of future investigations to unravel the mechanisms by which these proteins suppress and enhance immunity. Indeed, their involvement in both pro-inflammatory and anti-viral cytokine signalling may identify novel targets for treatment of both viruses and immune disorders.

Since this study highlights a novel role for these SOCS proteins, cell lines lacking SOCS4 and SOCS5 should be generated to further confirm their effects on the TNF-α and IL-6 signalling pathways. Mutation of the SH2 domain and the SOCS-box domain of SOCS4 and SOCS5 could also provide new insights into their mechanism of

action. It is possible that SOCS5, like SOCS3, interacts with TRAF2 and inhibits downstream IKK activation. Therefore, co-immunoprecipitation studies could help determine if SOCS5 targets TRAF2 or other intermediates of the TNF-α signalling pathway. Since It is also possible that SOCS5, like SOCS2, regulates another member of the SOCS family, SOCS3-/- MEFs cells could be utilized to determine if SOCS5 is still able to enhances IL-6-mediated STAT3 phosphorylation in cells lacking SOCS3. While our discoveries have revealed new and exciting roles for both SOCS4 and SOCS5 in immune signal transduction, future studies should further explore the precise mechanism by which these proteins regulate IL-6 and TNF-α signal transduction, which may indeed reveal novel targets for treatment of inflammatory disorders.

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