THE ROLE OF SUPPRESSOR OF CYTOKINE SIGNALING 1 (SOCS-1) MIMETIC AND ANTAGONIST PEPTIDES IN HOST DEFENSE

By

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To Angie and Claudio Dabelic, for encouraging my curiosity and indulging my desire for learning

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LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
BLAST	basic local alignment search tool
Btk	Bruton's tyrosine kinase
CIS	cytokine-inducible Src homology 2 containing protein
CLC	cardiotropin-like cytokine
CNTF	ciliary neurotrophic factor
CPE	cytopathic effect
CT-1	cardiotrophin 1
CTL	cytotoxic T cell
DC	dendritic cell
DMSO	dimethyl sulfoxide
dsDNA	double stranded DNA
EAE	experimental allergic encephalomyelitis
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
EMCV	encephalomyocarditis virus
EPO	erythropoietin
ESS	extended SH2 sequence
G-CSF	granulocyte colony-stimulating factor
GAF	interferon γ-activated factor
GAS	interferon γ-activated sequence
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor

HPLC	high performance liquid chromatography
HSV-1	herpes simplex virus 1
IFN	interferon
IFNAR	interferon α receptor
IFNGR	interferon γ receptor
IGF	insulin-like growth factor
IL	interleukin
i.p.	intraperitoneal
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulated response element
JAB	Janus kinase binding protein
JAK	Janus kinase
JH	JAK homology
KIR	kinase inhibitory region
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAL	MyD88 adapter-like
MHC	major histocompatibility complex
moi	multiplicity of infection
NF-κB	nuclear factor κB
NO	nitric oxide
OSM	oncostatin M

PAMP	pathogen-associated molecular pattern
pfu	plaque forming unit
PIAS	protein inhibitors of activated signal transducers and activators of transcription
Poly I:C	polyriboinosinic:polyribocytidylic acid
RBX2	really interesting new gene (RING) box 2
SCID	severe combined immunodeficiency
SH2	Src homology 2
SHP	SH2-containing tyrosine phosphatase
siRNA	small interfering RNA
SOCS	suppressor of cytokine signaling
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
TAK1	transforming growth factor β-activated kinase 1
TGFβ	transforming growth factor β
T_H1	T helper 1
T_H2	T helper 2
Tkip	tyrosine kinase inhibitor peptide
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
TPO	thrombopoietin
TRAF	tumor necrosis factor receptor-associated factor
TRAM	translocating chain-associating membrane protein
TRIF	Toll/interleukin-1 domain-containing adapter-inducing interferon β
TRP2	tyrosine-related protein 2

TSLP	thymic stromal lymphopoietin
TYK	tyrosine kinase
VSV	vesicular stomatitis virus

Abstract of Dissertation Presented to the Graduate School
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THE ROLE OF SUPPRESSOR OF CYTOKINE SIGNALING 1 (SOCS-1) MIMETIC AND ANTAGONIST PEPTIDES IN HOST DEFENSE

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Suppressors of cytokine signaling (SOCS) are negative regulators of both innate and adaptive immunity via inhibition of signaling by cytokines and Toll-like receptors. In this study, we report on the development of SOCS-1 mimetic peptides SOCS1-KIR and Tkip, and a SOCS-1 antagonist peptide pJAK2(1001–1013). The mimetic peptides imitate SOCS-1 by binding to the autophosphorylation sites of all of the Janus kinases (JAKs) and by inhibiting the activation of several signal transducers and activators of transcription (STATs). Moreover, they also directly inhibit Toll-like receptor (TLR) 4 signaling by inactivating the MyD88 adapter-like protein (MAL). Mice deficient in SOCS-1 die on average by 15 days of age, but when administered SOCS1-KIR they are able to survive to weaning. The SOCS-1 antagonist peptide inhibits the replication of vaccinia virus, herpes simplex virus 1 (HSV-1), and encephalomyocarditis virus (EMCV) in cell culture, suggesting that it possesses broad antiviral activity. In addition, pJAK2(1001–1013) increases the intracellular level of IFNβ and STAT1 phosphorylation, which may play a role in the antagonist antiviral effect at the cellular level. Antibody neutralization suggests that the endogenous IFNβ may act intracellularly. pJAK2(1001–1013) also synergizes with an IFNγ mimetic peptide,

IFNγ(95–132), to exert a multiplicative antiviral effect *in vitro* and *in vivo*. In addition to its antiviral properties, pJAK2(1001–1013) enhances TLR3 and TLR4 activation. The SOCS-1 antagonist presents a novel approach to enhancement of host defense against viruses, while the SOCS-1 mimetics present a way to control inflammation and may be potential therapeutics for autoimmune diseases such as multiple sclerosis.

CHAPTER 1
INTRODUCTION

Signaling Through the Janus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) Pathway

The ability of cells to respond to distinct stimuli from the environment is the key to the complexity of multicellular organisms. Cytokines and their receptors are the major players in this response (Borden et al., 2007). Many hormones and cytokines use the Janus kinase (JAK) family of kinases and the signal transducers and activators of transcription (STAT) family to initiate intracellular signaling (Table 1-1). A few examples are interferons (IFNs), some interleukins (ILs), erythropoietin (EPO), growth hormone, insulin, leptin, and lactin (O'Sullivan et al., 2007).

The current model for signaling through the JAK/STAT pathway (illustrated in Figure 1-1) involves a cytokine binding to its receptor(s), which activates the JAKs that are associated with their receptor subunits via JAK binding sites located proximal to the membrane (Miura et al., 1994; VanderKuur et al., 1994; DaSilva et al., 1994). The JAKs then phosphorylate the cytoplasmic domain of their receptors, creating docking sites for the Src homology 2 (SH2) domain of STATs. Once the STATs are recruited to the receptor, they are phosphorylated on key tyrosine and serine residues by JAKs and other kinases. This activation of the STATs allows them to form homo- or hetero-dimers that subsequently translocate to the nucleus to bind specific sequences on the genomic DNA to activate gene expression (reviewed in O'Shea et al., 2002).

The JAK Family of Kinases

The JAK family of cytoplasmic kinases consists of four members in the mammalian system: JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). They are differentially activated in response to various cytokines, and are expressed in many

types of tissue with the exception of JAK3, which is limited to cells of the hematopoietic system (Ihle, 1995). The JAKs are large tyrosine kinases (120–140 kDa) that are associated with the cytoplasmic regions of cytokine receptor subunits (Haan et al., 2006). They consist of seven highly conserved JAK homology (JH) domains (illustrated in Figure 1-2) (Wilks et al., 1991). The JH1 domain, located at the C-terminus, is a classical kinase domain that is required for kinase activity. It contains the activation loop that becomes phosphorylated at a critical tyrosine residue (Y), located around position 1000 in all four JAKs, which activates the kinase. This autophosphorylation site is critical for the activity of JAKs as well as for downstream signaling events (Feng et al., 1997). Phosphorylation of this tyrosine residue causes a conformational change in the activation loop, allowing substrate access to binding sites in the catalytic groove (Yasukawa et al., 1999). Comparison of the amino acid sequence of the autophosphorylation site of JAK2 (^{1001}LPQDKEYYKVKEP) showed 100% sequence identity among mammalian species including human, mouse, rat, and pig, as determined using the basic local alignment search tool (BLAST, http://www.ncbi.nlm.nih.gov/blast/). Among all four JAK kinases, the protein sequences of the autophosphorylation sites are very similar, highlighting the importance of the autophosphorylation site in JAK function.

Preceding the JH1 domain N-terminally is the JH2 domain, a pseudokinase domain thought to be involved in the autoregulatory activity of JAKs (reviewed in Haan et al., 2006). It has a classical kinase domain fold but lacks the residues required for catalytic activity and for nucleotide binding. There is an SH2 region spanning the JH3

and JH4 domains. The JH3–JH7 domains comprise the rest of the N-terminus, and are involved in cytokine receptor binding.

The JAK kinases are linked to signaling by cytokines of the hematopoietic system, including interleukins, colony-stimulating factors, interferons, erythropoietin, and thrombopoietin (reviewed in Khwaja, 2006). The majority of these cytokines bind to a family of transmembrane receptors, either monomeric or heterodimeric, that share structural features. The heterodimeric receptors share a common signaling subunit and a unique ligand-binding chain (reviewed in Rane and Reddy, 2002). These can further be grouped into receptors which share the common β-chain (granulocyte macrophage colony-stimulating factor, IL-3, IL-5), the gp130 subunit (IL-6, leukemia inhibitory factor, oncostatin M, IL-11), or the common γ-chain (IL-2, IL-4, IL-7, IL-9, IL-13, IL-15). The single chain and heterodimeric group together make up the type I cytokine receptors, which are characterized by the presence of a WSXWS motif, fibronectin type III domains in the extracellular part of the receptor, and by conserved Box1/Box2 regions in the membrane proximal cytoplasmic domain (Khwaja, 2006). The type II cytokine receptors include the interferon and IL-10 receptors, and they lack the WSXWS motif but do have the Box1/Box2 region (Khwaja, 2006). Signaling via these cytokine receptors is initiated by ligand binding, which induces the dimerization or a conformational change of receptor subunits. The JAKs are constitutively associated with the receptor subunits via their FERM domain and the receptor Box1 domain, thereby conferring the functional equivalent of a receptor tyrosine kinase, as these receptors lack this quality (Behrmann et al., 2004). Receptor oligomerization brings the associated JAKs to close proximity, allowing their auto- or trans-phosphorylation and activation.

The JAKs have a multitude of physiological roles, including regulating the cell surface expression of their associated receptors (Ragimbeau et al., 2003), and the recycling and degradation of their associated receptors (Gauzzi et al., 1997). Their uncontrolled activation has been implicated in numerous hematological malignancies, immunodeficiency syndromes, oncogenesis, myeloid proliferative disorders, and cardiovascular diseases (reviewed in Sandberg et al., 2004; Khwaja, 2006). Gene targeting studies of the JAK kinases in mice show distinct phenotypes. (Table 1-2) (reviewed in Igaz et al., 2001). JAK1 knockout mice die perinatally due to profound defects in lymphoid development (Rodig et al., 1998), while JAK2 knockout mice die in the embryonic period due to a lack of erythropoiesis (Parganas et al., 1998). This correlates with the role of JAK2 in erythropoietin (EPO) and thrombopoietin (TPO) signaling. Mice deficient in JAK3 are viable, owing to the limited expression of JAK3. They exhibit the murine severe combined immunodeficiency (SCID) phenotype that affects B- and T-cell populations. TYK2 knockout mice are phenotypically normal but have been shown to have exercise intolerance compared to their littermates, suggesting a mitochondrial respiration disregulation (Potla et al., 2006).

The STAT Family

STAT proteins are a family of latent cytoplasmic transcription factors that, when phosphorylated on a tyrosine residue by a member of the JAK family, dimerize via their SH2 domains and translocate to the nucleus where they activate gene transcription (reviewed in Darnell, 1997). There are seven known mammalian STAT family members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Each STAT is composed of five domains, including a four-helix bundle transactivation domain, a central β-barrel DNA binding domain, a helical linker domain, an SH2 domain, and an

effector domain. The helical linker domain forms a bridge between the DNA binding and SH2 domains. The C-terminal transactivation domain, involved in communication with transcription complexes, has a conserved serine residue (not found in STAT2 or STAT6) that, when phosphorylated, regulates STAT transcriptional activity (Kovarik et al., 2001). The effector domain is involved in regulatory function of the STATs and may be responsible for their nuclear export. The N-terminal region of the STATs is highly conserved and contributes to the stability of STAT–DNA binding, thereby increasing the transcriptional activity (reviewed in Imada and Leonard, 2000).

STATs bind to receptors via the interaction of their SH2 domains with their appropriate receptor-docking site. Phosphorylation of a single tyrosine residue located around amino acid residue 700 in each of the STATs is required for their activation. The proteins responsible for the phosphorylation can be cytokine receptors with intrinsic tyrosine kinase activity, or receptors that are associated with JAKs. Once they are phosphorylated, the STATs form homo- or heterodimers. This dimerization is required for DNA-binding activity, as monomers are not capable of binding DNA. The STATs that form heterodimers are STAT1:STAT2 and STAT1:STAT3, while the ones that form homodimers are STAT1, STAT3, STAT4, STAT5, and STAT6 (reviewed in Darnell, 1997).

The physiological role of the STATs has been widely investigated in a wide variety of experimental models, but due to the complexity of the interactions between and among the various JAKs and STATs, the most clear-cut results were determined using STAT knockout mice (reviewed in Khwaja, 2006). Table 1-3 summarizes their phenotypes. STAT1 deficient mice have no innate immune response to either bacterial

or viral infection, but if kept in a pathogen-free environment they are phenotypically normal and capable of reproducing (Meraz et al., 1996). Knocking out the STAT4 gene resulted in mice deficient in T helper 1 (T_H1) cell function. Likewise, STAT6 null mice had deficient T helper 2 (T_H2) cell function. This is supported by findings that STAT4 is activated in response to IL-12, a cytokine that drives T cells to the T_H1 phenotype (Kaplan et al., 1996), and that STAT6 is activated in response to IL-4, which promotes a T_H2 phenotype (Shimoda et al., 1996). Male mice deficient in STAT5A are phenotypically normal, while the female mice are not able to develop normal breast tissue and cannot lactate. Deficiency in STAT5B causes male mice to grow slowly, and their serum levels of liver-produced proteins are more characteristic of female mice. This sexual dimorphism is due to the activation of STAT5A and STAT5B by growth hormone (Udy et al., 1997). In addition, STAT5A and STAT5B deficiency in $CD4^+$ T cells results in a significant reduction of $CD4^+CD25^+Foxp3^+$ T cells in the thymus and periphery (Burchill et al., 2007; Yao et al., 2007). Further, humans with mutations in the STAT5A/B genes display immune disregulation that is associated with decreased CD25 and Foxp3 expression (Cohen et al., 2006). Finally, the IL-12Rβ chain-dependent activation of STAT5 is necessary for the development and homeostasis of T regulatory cells (Burchill et al., 2007).

STAT3 is expressed in the visceral endoderm, whose function is required for gastrulation. In accord with these findings, STAT3 knockout mice die before reaching the gastrulation phase. STAT2 deficiency is also embryonically lethal, but the exact stage has not been determined (Darnell, 1997).

Interferons

Interferons (IFNs) were first described over 50 years ago as agents that interfered with viral infection (Lindenmann et al., 1957). Since then, much work has been done on elucidating the wide variety of biological effects of the IFNs. This includes control over cell growth and tumors, as well as regulating both the innate and adaptive arms of the immune response (reviewed in Bonjardim et al., 2009). Interferons are now described as a family of secreted autocrine and paracrine proteins that serve to stimulate intracellular and extracellular networks that regulate resistance to viral infections, modulate normal and tumor cell survival and death, and enhance innate and adaptive immune responses (Borden et al., 2007).

The Interferon Family

There are three types of IFNs (reviewed in Bonjardim et al., 2009). The type I IFNs include IFNα (which in humans includes 13 members), β, ω, ε, κ, δ, and τ. These IFNs all have antiviral activity, share a high degree of homology in terms of protein sequence, and do not have introns. They signal through the type I IFN receptor subunits, IFNα receptor 1 and 2 (IFNAR1 and IFNAR2). Type II IFNs have only one member, IFNγ. It has antiviral activity like the type I IFNs, but also has an immunomodulatory function. IFNγ signals through the IFNγ receptor (IFNGR) subunits 1 and 2 (IFNGR1 and IFNGR2). A newly recognized IFN family is the type III IFNs, or IFNλs, which are also known as IL-28A, IL-28B, and IL-29. They signal through the IL-10 receptor β and IL-28 receptor α chains to control cell proliferation and exert their antiviral activity. Unlike the other IFNs, however, the type III IFNs contain introns (Kotenko et al., 2003).

Interferon Signaling Through the JAK/STAT Pathway

All IFNs are α-helical cytokines that signal through the JAK/STAT pathway. The type I IFNs use IFNAR-associated JAK1 and TYK2 to activate STAT1 and STAT2, which in combination with IFN-regulatory factor (IRF)-9 form the heterotrimeric transcription factor IFN-stimulated gene factor (ISGF)-3. ISGF-3 then translocates to the nucleus to bind to IFN-stimulated response elements (ISREs) in the DNA to promote gene expression. IFNγ uses IFNGR-associated JAK1 and JAK2 to activate STAT1, which dimerizes with itself to form the transcriptional regulator IFNγ-activated factor (GAF). The GAF recognizes elements in the DNA termed the IFNγ-activated sequences (GAS) and binds to them to initiate gene activation. The type III IFNs use JAK1 to activate STAT1, STAT2, and ISGF-3 to turn on the expression of IFN-stimulated genes (ISGs) that are also turned on upon type I IFN stimulation (reviewed in Bonjardim et al., 2009).

As previously mentioned, the current model of JAK/STAT signaling shows the cytokine-stimulated receptor activation, followed by auto- or transphosphorylation of the receptor-associated JAKs, which also phosphorylate the receptor subunits to create docking sites for the SH2 regions of the STATs, causing them to form homo- or heterodimers and translocate to the nucleus, where they activate gene expression. The IFN signaling pathway is illustrated using this same model, where the only function of the cytokines, receptors, and JAKs is to turn on the relevant STATs so they can activate gene expression. Recent studies have shown that, in the case of type II IFNs, the IFNGR1 and IFNGR2 receptor subunits come together upon stimulation with IFNγ, IFNγ moves to the cytoplasm, followed by activation of JAK1, JAK2, and STAT1. The

IFNGR1 receptor subunit, IFNγ, and STAT1 homodimer are subsequently translocated to the nucleus as a complex (Subramaniam et al., 2001; Ahmed et al., 2003; Johnson, 2004).

Regulation of Cytokine Signaling

Cytokine signaling has to be tightly regulated, as unregulated signaling could result in unwanted inflammation and/or autoimmune diseases that are harmful to the organism. There are multiple regulatory pathways to contain cytokine signaling, including activation of tyrosine phosphatases, receptor internalization, proteasomal degradation of signaling adapter molecules, soluble receptor agonists (usually employed by viruses), and specific inhibitors of cytokine signaling (Croker et al., 2008). These inhibitors are important not only for keeping the immune response in check, but also for the differentiation of the cells that are the responders of the immune system (Yoshimura et al., 2007).

There are currently three known classes of regulators of the JAK/STAT pathway. They are the protein inhibitors of activated STATs (PIAS), the SH2-containing tyrosine phosphatases (SHPs), and the suppressors of cytokine signaling (SOCS). PIAS proteins regulate transcription through multiple mechanisms, one of which is blocking the DNA-binding ability of transcription factors (Shuai, 2006). SHPs are phosphatases that dephosphorylate activated phosphotyrosine-containing proteins, such as JAKs and STATs (Lorenz, 2009). SOCS can regulate the JAK/STAT pathway in multiple ways, which are discussed in detail below.

Suppressors of Cytokine Signaling (SOCS)

The discovery of the SOCS proteins was published concurrently by three groups: as a JAK binding protein (JAB) (Endo et al., 1997), as a suppressor of IL-6 signaling

(Starr et al., 1997), and based on sequence homology with the STAT3 SH2 domain (Naka et al., 1997). The SOCS proteins have been shown to regulate over 30 different cytokines, including IL-6, leukemia inhibitory factor (LIF), IL-10, growth hormone, IFNγ, and most recently IL-17 and IL-23 (Table 1-4) (reviewed in Croker et al., 2008)

SOCS Structure and Function

There are currently eight members of the SOCS family: SOCS-1 through 7 and cytokine-inducible SH2-containing protein (CIS). They each have a central SH2 domain, an N-terminal domain of variable length and sequence, and a C-terminal SOCS box motif (Figure 1-3). The SOCS box interacts with elongins B and C, cullin-5, and RING-box-2 (RBX2), which recruits the E2 ubiquitin transferase, allowing the SOCS proteins to act as E3 ubiquitin ligases, which mediate the proteasomal degradation of proteins that are associated with them (Kamura et al., 2004). The structure of the SOCS box of SOCS-3 in complex with elongins B and C has recently been solved (Babon et al., 2008), as has the partial structure of SOCS-3 in complex with a peptide from the IL-6 receptor gp130 subunit (Bergamin et al., 2006). These studies showed that there is a great deal of disorder in the SOCS box of SOCS-3 when it is not bound to a ligand, and that it is this flexibility that is a key feature of the interaction between SOCS-3 and its various ligands.

SOCS-1 and SOCS-3 (and possibly SOCS-5 [Croker et al., 2008]) have an N-terminal kinase inhibitory region (KIR) that is important for their inhibitory effects on kinases such as JAK2 (Waiboci et al., 2007; Ahmed et al., 2009). The KIR consists of a 12 amino acid region that interacts specifically with the activation loop of JAK2 to inhibit its kinase activity (Waiboci et al., 2007; Ahmed et al., 2009). The importance of this

region in the binding of JAKs has been debated, with most of the binding being ascribed to the SH2 region.

The SH2 region of the SOCS-1 and SOCS-3 proteins is preceded N-terminally by a short sequence termed the extended SH2 sequence (ESS), and it has been shown to be critical in the binding of phosphotyrosine residues. It forms a 15-residue α-helix that has been shown to directly contact the phosphotyrosine-binding loop and determines its orientation, providing structural integrity to the SOCS molecule (Sasaki et al., 1999; Babon et al., 2006). The central SH2 region of the SOCS proteins binds to phosphorylated tyrosine residues found on many proteins, including the IFNGR1 and IFNAR1 receptor subunits in the case of SOCS-1 (Fenner et al., 2006; Qing et al., 2005), and the gp130 (Schmitz et al., 2000) and IL-12Rβ2 receptor subunits in the case of SOCS-3 (Yamamoto et al., 2003). The regulation of receptor phosphorylation by SOCS would allow for the suppressive effect on cytokine signaling even in the presence of low amounts of SOCS proteins (Yoshimura et al., 2007).

The Physiological Role of SOCS-1

SOCS-1 is necessary for survival, as SOCS-1 knockout mice die as neonates due to low body weight, multi-organ inflammation, necrosis of the liver, and monocytic infiltration of the pancreas, lung, and heart. In addition, their thymuses are severely reduced in size and they have a deficiency in mature B and T lymphocytes (Starr et al., 1997; Naka et al., 1997).

The pathology in the SOCS-1 knockout mice is similar to that observed in wild-type mice treated with excess IFNγ. This is complemented with the observation that SOCS-1$^{-/-}$IFN$\gamma^{-/-}$ mice did not show the lethal phenotype of the SOCS-1$^{-/-}$ mice. In

addition, SOCS-1$^{-/-}$ mice treated from birth with neutralizing antibodies to IFNγ for three weeks remained viable and healthy while their untreated counterparts succumbed to disease (Alexander et al., 1999). These observations led to the conclusion that SOCS-1 is a key regulator of IFNγ signaling. However, the SOCS-1$^{-/-}$IFNγ$^{-/-}$ mice eventually died by 6 months of age due to inflammation and polycystic kidneys, which would suggest that the regulatory abilities of SOCS-1 are not limited to IFNγ (Metcalf et al., 2002). The regulation of IFNγ by SOCS-1 was confirmed by injecting IFNγ into SOCS-1$^{-/-}$ mice and looking for STAT1 phosphorylation levels. In wild-type mice, phosphorylated STAT1 was found in the liver 15 minutes after treatment, and it declined 2 hours after treatment. In the SOCS-1$^{-/-}$ mice, phosphorylation of STAT1 was evident 8 hours after treatment, suggesting continuous, unregulated IFNγ signaling (Brysha et al., 2001). It has additionally been shown that SOCS-1 is also a regulator of type I IFN signaling. Using neutralizing antibodies to IFNα and IFNβ and mice deficient in IFNγ, IFNAR1 or IFNAR2, Fenner et al. (2006) demonstrated that SOCS-1 deficiency amplified type I IFN antiviral actions independently of IFNγ.

The Role of SOCS-1 in Innate Immunity

Toll-like receptors (TLRs) are key players in the immune response to invading pathogens. There are over 10 recognized TLRs in humans and mice. They recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) or double-stranded DNA (dsDNA). These signals must be tightly regulated to avoid excessive inflammation and damage to the host (reviewed in Carpenter and O'Neill, 2009). TLR ligands such as LPS and CpG-containing DNA are potent inducers of SOCS-1 (reviewed in Yoshimura et al., 2007).

Mice deficient in SOCS-1 are hyper-responsive to LPS and hypersensitive to LPS-induced lethality (Nakagawa et al., 2002). *In vivo* treatment with LPS results in a strong production of IFNγ, IL-12, and nitric oxide (NO) (Kinjyo et al., 2002). Several mechanisms for the regulatory activity of SOCS-1 on TLRs have been proposed and a few are shown in Figure 1-4. One mechanism is through SOCS-1 binding to p65 of nuclear factor κB (NF-κB) and mediating its proteasomal degradation via the SOCS box (Ryo et al., 2003). SOCS-1 has been shown to bind to tyrosine phosphorylated MyD88 adapter-like (MAL) protein and induce its proteasomal degradation (Mansell et al., 2006). MAL is an adapter protein that is used by MyD88-dependent TLR signaling pathways, specifically, those activated by TLR2 and TLR4 stimulation. After stimulation by TLR ligands, the MyD88-mediated signaling pathway leads to activation of NF-κB and induction of IFNβ and other inflammatory gene products. The induction of IFNβ leads to activation of JAK/STAT signaling, which is also regulated by SOCS-1 (Fenner et al., 2006). TLR activation is extremely important in pathogen clearance, but excessive activation can lead to harmful pathogenesis to the host. It is therefore important to be able to regulate overactive signaling in order to prevent fatal responses to systemic infection.

The Role of SOCS-1 in Infection

SOCS-1 is an important mediator of IFN signaling. As such, it contributes to the balance of beneficial antiviral and detrimental pro-inflammatory effects of IFN signaling. It has been shown that SOCS-$1^{-/-}$ mice are resistant to viral infection (Fenner et al., 2006). These mice responded to type I IFNs for a longer amount of time to clear virus more efficiently and therefore survive a lethal viral infection. In addition, the expression

of a dominant negative form of SOCS-1 in cardiac myocytes increased their resistance to enteroviral infection (Yasukawa, 2003). These data collectively indicate that low SOCS-1 protein levels are important for host defense against viral infection through their regulation of type I and II IFNs.

SOCS-$1^{-/-}$ mice are not only resistant to viral infection, but certain parasitic infections as well. SOCS-1 is directly induced by *Toxoplasma gondii* parasites, and is involved in IFNγ-inhibition in infected cells (Zimmermann et al., 2006). The induction of SOCS-1 is therefore most likely a strategy to evade the immune response.

Specificity of Cytokine Signaling

With only four receptor-associated JAK kinases and seven STATs being involved in the signaling of over 40 different cytokines and hormones, how is specificity of gene activation achieved? Further, we still do not understand the exact interactions between the JAKs and their receptors, and between JAKs and STATs, in terms of protein structure. The exact sequence of events from ligand binding to the receptor, to the phosphorylation of the JAKs, to the recruitment and subsequent nuclear translocation of STATs, is still not clear. We do know that the JAK-mediated phosphorylation of the receptor subunits creates docking sites on the receptor for the SH2 domain of the relevant STAT, and that this recruitment is followed by phosphorylation of critical tyrosine and serine residues on the STAT proteins. From here, the events are yet to be clearly understood.

There are several theories on what happens after the STATs are recruited to the receptors. The “classical” model of cytokine signaling has the STATs forming homo- or heterodimers and then translocating to the nucleus by themselves in order to activate

gene expression (reviewed in Borden et al., 2007). Recent studies in our lab based on signaling by IFNγ have proposed another model that is currently being tested using other cytokines that signal through the JAK/STAT pathway (Ahmed et al., 2003). Binding of IFNγ to the IFNGR1 and IFNGR2 receptor subunits causes the activation of JAK1 and JAK2 and induces binding of STAT1 to IFNGR1. The binding of IFNγ additionally initiates the selective endocytosis of IFNGR1 and the associated proteins JAK2 and STAT1 homodimer. Interestingly, IFNγ is found bound to the cytoplasmic domain of the IFNGR1 subunit, allowing for its C-terminal nuclear localization sequence (NLS, ^{126}RKRKRSR) to be exposed and recognized by the nuclear importin machinery, allowing the entire complex to be translocated to the nucleus, where the complex binds to the GAS region of the IFNγ promoter. This model ascribes roles to the ligand and receptor beyond that of signal transduction initiators. As such, replacing the basic residues of IFNγ NLS with alanines and expressing the mutant intracellulary, failed to induce nuclear translocation of IFNGR1 or STAT1, and led to a loss of IFNγ activities (Ahmed et al., 2003). Further, in cells expressing the IFNγ NLS mutant, STAT1 was not translocated to the nucleus, even though it was phosphorylated and activated (Ahmed et al., 2003), suggesting that an important function of IFNγ in this model is to act as a chaperone for the nuclear import of activated STAT1.

Another theory involves the tissue-specific expression of cytokine receptors. Different tissues and cell types express distinct receptor combinations unique to their microenvironment, allowing the cells to integrate signals from multiple cytokine receptors. The complication here is that cytokine receptors preferentially associate with certain JAKs. For example, the IFNGR complex only associates with JAK1 and JAK2

(O'Shea et al., 2002). This raises several questions: with over 40 different cytokines using only four JAKs, and their only function is to phosphorylate tyrosine residues on the receptors and STATs, why are the JAKs not interchangeable? Does one cytokine activate the same genes in every cell type that is responsive to it? What role do negative regulators of cytokine signaling, such as the SOCS proteins, play in the specificity of signaling? The complexity of the subject is increased when the interactions among the different pathways are taken into account. It has been shown that some functions of the STATs are redundant, i.e. if one STAT is knocked out, another STAT can compensate (Gil et al., 2001; Ramana et al., 2001). Additionally, SOCS proteins such as SOCS-1 and SOCS-3 are induced by STATs, and while they have similar mechanisms of action, they are activated in a cytokine-specific fashion (Murray, 2007). Further, certain cytokines can induce the expression of receptors, co-receptors, or adapter molecules during a "priming phase" (Bezbradica and Medzhitov, 2009), enabling the cell to respond to the cytokine for a prolonged period. This, in turn, further upregulates the expression of the negative regulators of cytokine signaling, like SOCS, which are able to modify the output of activated STATs and therefore alter downstream gene activation. Although the specificity of signaling is being intensively studied, there is still a large gap of knowledge in this field. With some of the results presented in this study and additional experiments being performed, we hope to fill some of these gaps.

Small Molecule Mimetic and Antagonist Peptides

SOCS-1 Mimetic Peptides

Inflammatory diseases in which cytokine signaling is hyperactive could be treated using SOCS-1 proteins or SOCS-1-like molecules (mimetics). Hyperactive pro-inflammatory cytokine signaling has been implicated in the pathogenesis of autoimmune

diseases such as multiple sclerosis, rheumatoid arthritis, and inflammatory diseases of the gastrointestinal tract. The current treatment options for inflammatory and autoimmune diseases are limited to the treatment of symptoms, not the actual disorder itself.

Our group has designed two SOCS-1 mimetic peptides, SOCS1-KIR and tyrosine kinase inhibitor peptide (Tkip). The first peptide to be made, Tkip, was designed to be complementary to the autophosphorylation site of JAK2, which encompasses residues 1001-1013 and contains the important phospho-tyrosine 1007 that is required for JAK2 kinase activity. As such, Tkip has been shown to bind to the JAK2 autophosphorylation site peptide, as well as inhibit JAK2 autophosphorylation and the JAK2-mediated phosphorylation of the IFNGR1 subunit. Tkip also inhibited the ability of IFNγ to upregulate major histocompatibility complex (MHC) I, and the ability to induce an antiviral state. In addition to its regulatory activity of IFNγ, Tkip was shown to inhibit epidermal growth factor receptor (EGFR) autophosphorylation (Flowers et al., 2004). Tkip was also shown to protect mice from experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis (Mujtaba et al., 2005). These data collectively would suggest that Tkip is able to act as a SOCS-1 mimetic, and that it has the potential to be used as a therapeutic treatment in autoimmune diseases such as multiple sclerosis.

The SOCS1-KIR peptide contains the 12-residue sequence that makes up the kinase inhibitory region of SOCS-1. It has been shown to bind to the autophosphorylation site peptide of JAK2 in a similar, but not identical, way to Tkip. SOCS1-KIR was also shown to inhibit the IFNγ-induced STAT1 phosphorylation in a

manner similar to Tkip, the IFNγ-induced activation of macrophages and antigen-specific splenocyte proliferation, and EGFR autophosphorylation, similar to Tkip (Waiboci et al., 2007). As for Tkip, these data suggest that SOCS1-KIR acts as a SOCS-1 mimetic.

In this study, we will show that SOCS1-KIR and Tkip are able to act as SOCS-1 mimetics. They are able to bind to and regulate the activity of all four JAKs, and they are able to decrease or inhibit the activation of STATs in response to cytokines that use the type II cytokine receptors. In addition, the SOCS-1 mimetics are able to decrease macrophage activation in response to TLR ligands such as lipopolysaccharide (LPS). Further, SOCS1-KIR is able to partially remedy the pathophysiology of SOCS-1 knockout mice and prolong their survival.

SOCS-1 Antagonist Peptide

The ability of SOCS1-KIR to bind to the autophosphorylation site peptide of JAK2, pJAK2(1001–1013), would suggest that pJAK2(1001–1013) has the ability to act as a SOCS-1 antagonist. LNCaP cells overexpressing SOCS-1 showed a reduced level of IL-6-induced STAT3 phosphorylation that was increased 2-fold when treated with pJAK2(1001–1013) peptide. In addition, pJAK2(1001–1013) was able to enhance IFNγ function at the level of gene activation, as well as the antiviral activity of IFNγ against encephalomyocarditis virus (EMCV) in mice (Waiboci et al., 2007). Therefore, pJAK2(1001–1013) can be classified as a SOCS-1 antagonist. This has implications for treatments of diseases and infections where an increase in IFN signaling and decrease in SOCS-1 protein levels would be advantageous in order to clear an invading pathogen such as *T. gondii*, which has been shown to induce SOCS-1 in order to evade the

immune response (Zimmermann et al., 2006), or viruses that have similar immune evasion strategies.

In this study, we will show that pJAK2(1001–1013) is able to activate STAT1 in the absence of added cytokine stimulation, and that it is able to increase the activation of macrophages in response to TLR ligands that are encountered during bacterial or viral infection. In addition, pJAK2(1001–1013) is able to increase levels of endogenous IFNβ while simultaneously decreasing levels of SOCS-1. Further, pJAK2(1001–1013) can be used to protect cells and mice from infection by various viruses, suggesting that it can be used as a broad spectrum antiviral.

IFNγ Mimetic Peptide

With the studies performed in our lab on the signaling of IFNγ, we have identified the C-terminal domain of IFNγ as having biological function, using a synthetic peptide approach (Ahmed et al., 2003). We made a peptide corresponding to a portion of the C-terminal portion of murine IFNγ, spanning residues 95–132, IFNγ(95–132). It has been shown to bind to a soluble IFNGR1 chain that lacked the transmembrane domain (Johnson et al., 2004), suggesting a possible interaction with the cytoplasmic portion of the receptor subunit. This was confirmed by synthesizing overlapping peptides corresponding to the cytoplasmic portion of IFNGR1 and testing the binding ability of IFNγ(95–132) (Subramaniam et al., 2001). The IFNγ(95–132) peptide was further shown to have IFNγ-like biological activity, including upregulation of MHC class I expression, and reduction of vesicular stomatitis virus (VSV) titer (Szente and Johnson, 1994; Szente et al., 1994). In addition, the IFNγ(95–132) peptide was able to form a complex with IFNGR1, STAT1, and the nuclear import machinery (Subramaniam et al., 2000).

Collectively, these data suggest that IFNγ(95–132) can function as an IFNγ mimetic. In this study, we show that IFNγ(95–132) can synergize with pJAK2(1001–1013) to protect cells and mice from viral infection.

Table 1-1. Ligands that signal using Janus kinases (JAKs) and signal transducers and activators of transcription (STATs).

Ligands	JAKs	STATs
Erythropoietin	JAK2	STAT5
Growth Hormone	JAK2	STAT3, STAT5
Thrombopoietin	JAK2	STAT5
IL-2	JAK1, JAK3	STAT3, STAT5
IL-4	JAK1, JAK3	STAT6
GM-CSF	JAK2	STAT5
IL-6	JAK1, JAK2, TYK2	STAT1, STAT3
LIF	JAK1, JAK2, TYK2	STAT1, STAT3, STAT5
G-CSF	JAK1, JAK2, TYK2	STAT3
Leptin	JAK2	STAT3, STAT5, STAT6
IL-12	JAK2, TYK2	STAT1, STAT3, STAT4, STAT5
IL-23	JAK2, TYK2	STAT1, STAT3, STAT4, STAT5
Type I IFNs	JAK1, TYK2	STAT1, STAT2, STAT3-6
IFNγ	JAK1, JAK2	STAT1
IL-10	JAK1, TYK2	STAT1, STAT3, STAT5
IL-11	JAK1, JAK2, TYK2	STAT3
IL-3	JAK2	STAT3, STAT5
IL-5	JAK2	STAT1, STAT3, STAT5
Angiotensin	JAK2, TYK2	STAT1, STAT2, STAT3
Serotonin	JAK2	STAT3
Thrombin	JAK2	STAT1, STAT3
IL-7	JAK1, JAK3	STAT3, STAT5
IL-9	JAK1, JAK3	STAT1, STAT3, STAT5
Insulin	JAK1	STAT1, STAT5

Adapted with modifications from Schindler, 2002.

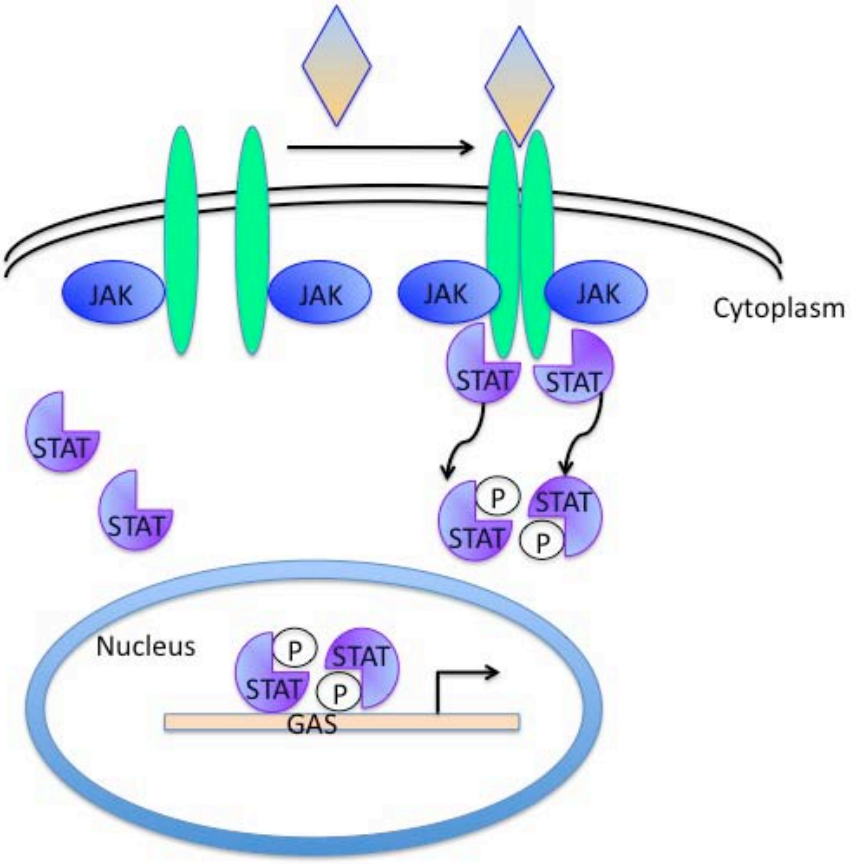

Figure 1-1. Model of the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) signaling pathway. A ligand binds to its receptor(s), activating the JAKs that are associated with their receptor subunits. The JAKs then phosphorylate the cytoplasmic domain of their receptors, creating docking sites for the SH2 domain of STATs. Once the STATs are recruited to the receptor, they are phosphorylated on key tyrosine and serine residues by JAKs and other kinases. This activation of STATs allows them to form homo- or hetero-dimers that subsequently translocate to the nucleus to bind specific sequences on the genomic DNA to activate gene expression.

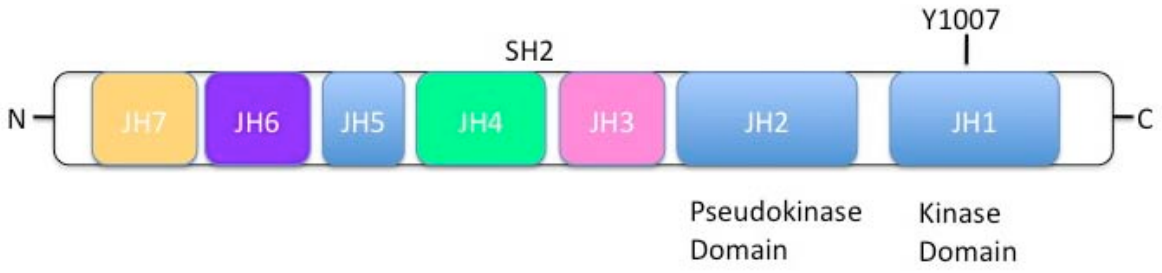

Figure 1-2. The JAK homology domains. The JH1 domain is a classical kinase domain that contains Y1007 of JAK2, required for kinase activity. The JH2 domain is a pseudokinase domain. There is an SH2 region spanning the JH3 and JH4 domains. The JH3–JH7 domains comprise the rest of the N-terminus, and are involved in cytokine receptor binding. Adapted with modifications from Imada and Leonard, 2000.

Table 1-2. Phenotypes of JAK knockout mice.

Targeted Gene	Phenotype
JAK1	Die perinatally, small at birth, no nursing
JAK2	Embryonic lethal, no erythropoiesis
JAK3	SCID
TYK2	Normal phenotype

Adapted with modifications from Igaz et al., 2001.

Table 1-3. Phenotypes of STAT knockout mice.

Targeted Gene	Phenotype
STAT1	No innate response to viral or bacterial infection
STAT2	Viable and fertile, defective type I IFN functions
STAT3	Early embryonic lethal
STAT4	No T_H1 function
STAT5A	No breast development or lactation
STAT5B	No breast development or lactation
STAT6	No T_H2 function

Adapted with modification from Darnell, 1997.

Table 1-4. Cytokines regulated by suppressors of cytokine signaling (SOCS).

SOCS	Regulates
CIS	Growth hormone, prolactin, IL-3
SOCS-1	IFNα, IFNβ, IFNγ, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-17, prolactin, erythropoietin, OSM, TSLP, TNFα, TPO, LIF, LPS
SOCS-2	Growth hormone
SOCS-3	IL-1, TGFβ, IL-6, IL-10, IL-11, IL-12, IL-17, IL-23, IL-27, G-CSF, leptin, LIF, OSM, CT-1, CNTF, CLC
SOCS-4	EGFR
SOCS-5	IL-4, EGFR
SOCS-6	Insulin
SOCS-7	Insulin, IGF

Adapted with modifications from Croker et al., 2008.

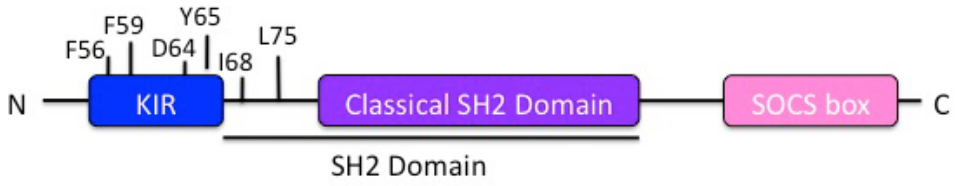

Figure 1-3. Structure of the suppressor of cytokine signaling (SOCS) proteins. The function of the SOCS box is to recruit the ubiquitin-transferase system. SOCS-1 and SOCS-3 contain a kinase inhibitory region (KIR) immediately upstream of the central SH2 domain, which inhibits the catalytic activity of JAKs by binding to the activation loop. Point mutations in this region completely abolish the suppressive effect of SOCS-1 and SOCS-3 on cytokine signaling. Adapted with modifications from Yoshimura et al., 2007.

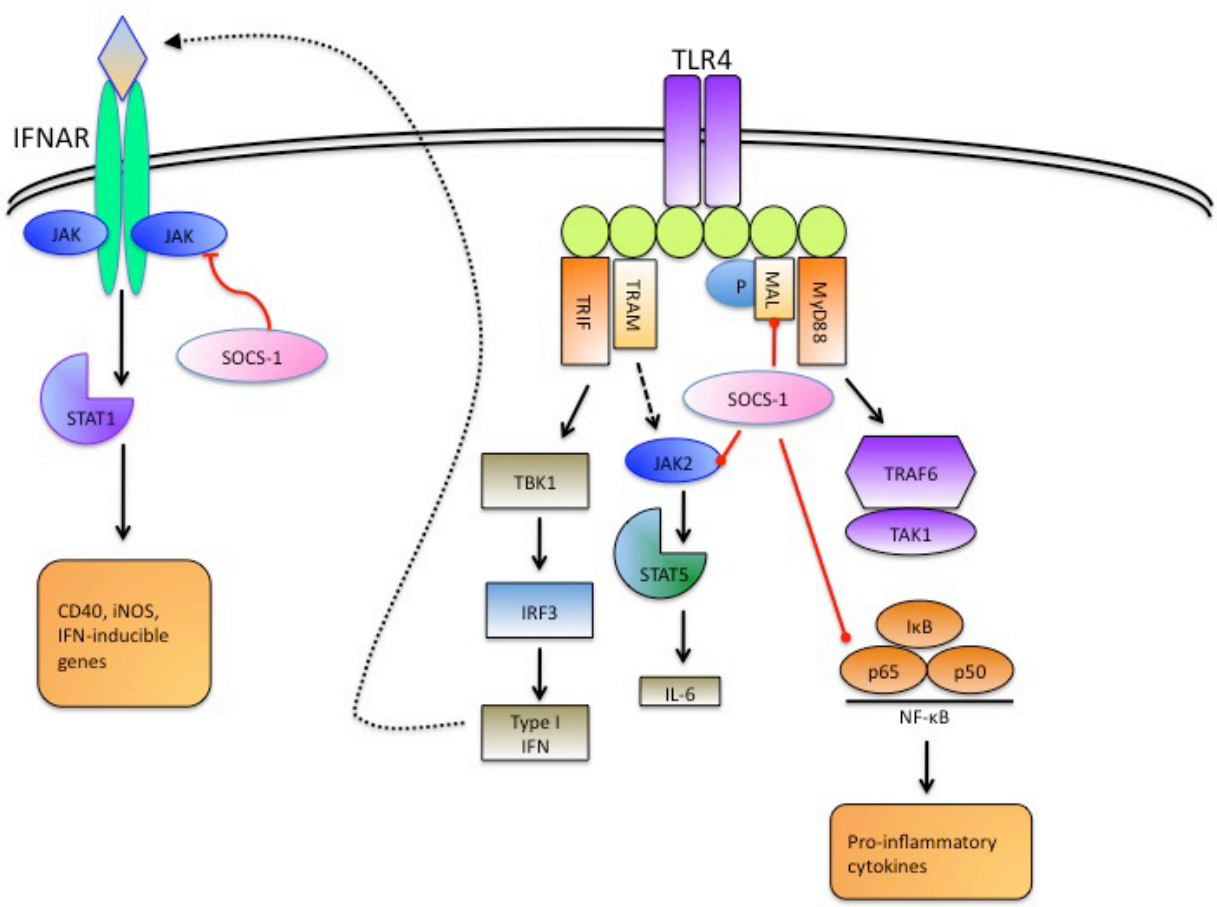

Figure 1-4. Mechanisms for the regulatory activity of SOCS-1 on Toll-like receptors (TLRs). Activation of TLR4 by LPS transmits signals through adapter proteins MyD88, MAL, TRIF and TRAM. NF-κB is activated by TRAF6 and TAK1 through MyD88 and MAL, whereas IRF3 is activated by TRIF and TRAM. IFNβ is rapidly induced through the TRIF/IRF3 pathway and activates the JAK/STAT pathway. The JAK2/STAT5 pathway has also been shown to be activated by LPS and is responsible for IL-6 production. Phosphorylated MAL interacts with SOCS-1, which results in MAL inactivation, ubiquitination and subsequent degradation. SOCS-1 also binds to the p65 subunit of NF-κB and induces its degradation. Adapted with modifications from Yoshimura et al., 2007.

CHAPTER 2
MATERIALS AND METHODS

Peptide Synthesis

The peptides used in this study were synthesized on an Applied Biosystems 431A automated peptide synthesizer (Applied Biosystems, Carlsbad, CA), using conventional fluorenylmethyloxycarbonyl (f-moc) chemistry, as previously described (Szente et al., 1994). To ensure cell penetration, a lipophilic palmitoyl-lysine was added to the N-terminus of each peptide used for cell culture or animal studies, using a semi-automated protocol (Thiam et al., 1999). The peptides were characterized by mass spectrometry, purified as needed by high-performance liquid chromatography (HPLC), and dissolved in dimethyl sulfoxide (DMSO) or phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO). The sequences of the peptides used in this study are presented in Table 2-1.

Cell Culture and Virus

L929, WISH, RAW264.7, and HEL-30 cells were obtained from ATCC (Manassas, VA) and propagated on DMEM with 10% fetal bovine serum (L929, WISH), EMEM with 10% calf serum (HEL-30), or RPMI 1640 with 10% fetal bovine serum (RAW264.7) supplemented with penicillin/streptomycin. All cells were grown at 37°C in humidified atmosphere with 5% CO_2. Encephalomyocarditis virus (EMCV) was grown and titrated on L929 cells, as described (Mujtaba et al., 2006). Herpes simplex virus 1 (HSV-1) was a kind gift from Dr. Nancy Bigley (Wright State University, Dayton, OH).

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida. All mice were housed in standard

specific pathogen free (SPF) facilities. For viral infections, female C57BL/6 mice (6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Peptides diluted in PBS in a volume of 100 μl were administered i.p. Fifty pfu of EMCV were diluted into PBS in a volume of 100 μl and administered i.p. Mice were monitored twice per day for signs of disease, such as lethargy, ruffled hair, weight loss, and eye secretions. Moribund mice were euthanized.

SOCS-$1^{+/-}$ mice were purchased from St. Jude Children's Hospital (Memphis, TN). Peptides were diluted in PBS and 10 μg of peptide per gram of body weight was administered daily i.p. in a volume of 30 μl for neonates, and 100 μl for adults. The mice were weighed daily and monitored for signs of inflammation.

Binding Assays

Peptides (3 μg) were bound to 96-well plates in binding buffer (0.1 M sodium carbonate-sodium bicarbonate, pH 9.6). The wells were washed three times with wash buffer (0.9% NaCl and 0.05% Tween-20) and incubated in blocking buffer (2% gelatin and 0.05% Tween-20 in PBS) for 1 h at room temperature. Then, the wells were washed three times with wash buffer and incubated with various concentrations of biotinylated peptides for 1 h at room temperature. The wells were washed five times with wash buffer and bound biotinylated peptides were detected using HRP-conjugated neutravidin (Invitrogen, Carlsbad, CA) and *o*-phenylenediamine in stable peroxidase buffer (Pierce Biochemicals, Rockford, IL). The chromogenic reaction was stopped by the addition of 2 M H_2SO_4 (50 μl) to each well. Absorbance was measured at 490 nm using a microplate reader (Bio-Tek, Winooski, VT).

Toxicity Studies

Cell viability experiments were performed on murine L929 cells and splenocytes isolated from healthy SJL/J mice using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI). Cells were plated out to confluence, and then incubated at 37°C with 5% CO_2 with medium or various peptides. After 24 h, the Solution Reagent was added and the cells were incubated for 2 h at 37°C, and the absorbance was measured at 490 nm using a microplate reader (BioTek, Winooski, VT).

Western Blot Analysis

Cells were incubated with various peptides or cytokines. The cells were washed in cold PBS and harvested in RIPA buffer containing protease and phosphatase inhibitor cocktails (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Protein concentration was measured using a BCA kit (Pierce Biochemicals, Rockford, IL) and lysates were resolved with SDS-PAGE, transferred onto nitrocellulose membranes, and probed with various antibodies. Detection of proteins was accomplished using ECL Protein Detection Reagents (Amersham Biosciences, Piscataway, NJ).

Macrophage Activation

Murine macrophages (RAW264.7) were seeded on 24-well plates at a concentration of 3×10^5 cells/well and allowed to adhere. Varying concentrations of peptides were then added to the cells and incubated for 2 h at 37°C in 5% CO_2. Purified lipopolysaccharide (LPS) at 2 μg/ml, or poly I:C (Sigma-Aldrich, St. Louis, MO) at 0.1 μg/ml were then added and incubated for an additional 48 h at 37°C. Supernatants were transferred into fresh tubes and assayed for nitrite levels as a measure of nitric

oxide production using Griess reagent according to manufacturer's instructions (Alexis Biochemicals, Plymouth Meeting, PA).

Interferon β ELISA

L929 cells were treated with peptides for 30 or 60 min and then lysed with RIPA lysis buffer containing protease inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). The cell lysates were analyzed with a murine IFNβ ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ), following manufacturer's instructions. Briefly, cell lysates were plated onto plate strips for 1 h at room temperature. The strips were washed three times with wash buffer, and then incubated with the Antibody Solution for 1 h at room temperature. After washing the strips three times, they were incubated with the HRP Solution for 1 h at room temperature. The strips were washed three times and incubated with TMB Substrate Solution for 15 minutes at room temperature. The reaction was stopped by addition of Stop Solution, and the absorbance was measured at 450 nm with a standard plate reader (BioTek, Winooski, VT).

Titration of Virus

HEL-30 keratinocytes were seeded onto 6-well plates at 2×10^4 cells/well and grown to confluence. Various peptides were added to the cells for 24 h, followed by removal of peptides and washing of cells with PBS. HSV-1 was then added to the cells at a multiplicity of infection (moi) of 0.1 for 1 h at 37°C. Then the virus was removed, the cells were washed, and fresh maintenance media was added. The cells were incubated for 2 days at 37°C in 5% CO_2. The media were collected and the viral titer was determined using a standard plaque assay protocol.

Antiviral Assays

Murine L929 cells were seeded in a 96-well plate at a cell density of 6×10^4 cells/well, and grown to confluence. Various concentrations of peptides were added and incubated for 2 h, after which 200 pfu of EMCV was added and incubated for 1 h. Then, the virus was removed, the cells were washed with PBS, and fresh maintenance media was added. After 24 h of incubation at 37°C, 5% CO_2, the cells were washed and stained with 0.1% crystal violet. Unbound crystal violet was removed and the plates were thoroughly rinsed with deionized water, blotted, and allowed to air dry. The plates were then scanned and analyzed using ImageJ 1.29 software (National Institutes of Health, Bethesda, MD) to assess cell survival. Percentages of cell survival were determined by comparing survival for the experimental treatment groups with that for the virus-only control group.

Statistical Analysis

Statistical differences between groups were determined by ANOVA with multiple comparisons. The Student's *t* test was used for comparisons when only two parameters were evaluated. Binding and proliferation assays were analyzed using the Mann-Whitney *U* test. p values < 0.05 were considered significant. Mouse survival data are presented as Kaplan-Meier plots, and analyzed using the log-rank test. Experimental data were measured for statistical significance using the GraphPad Prism software from GraphPad Software, Inc., San Diego, CA.

Table 2-1. Peptides used in this study.

Peptide Name	Sequence
Tkip	WLVFFVIFYFFR
Tkip2A	WLVFFVIAYFAR
SOCS1-KIR	^{53}DTHFRTFRSHSDYRRI
SOCS1-KIR2A	^{53}DTHFATFASHSDYRRI
JAK1 autophosphorylation site	^{1028}IETDKE**Y**YTVKDD
JAK2 autophosphorylation site	^{1001}LPQDKE**Y**YKVKEP
JAK3 autophosphorylation site	^{974}LPLDKD**Y**YVVREP
TYK2 autophosphorylation site	^{1048}VPEGHE**Y**YRVRED
MAL(82–94)	^{82}WSKD**Y**DVCVCHSE
MAL(154–166)	^{154}DPWCK**Y**QMLQALT
pJAK2(1001–1013)	^{1001}LPQDKE**Y**YKVKEP
JAK2(1001–1013)2A	^{1001}LPQDKEAAKVKEP
IFNγ(95–132)	^{95}AKFEVNNPQVQRQAFNELIRVVHQLLPESSL
IFNγ(95–125)	^{95}AKFEVNNPQVQRQAFNELIRVVHQ
IFNγ(95–106)	^{95}AKFEVNNPQVQR
IFNGR1(253–287)	^{253}TKKNSFKRKSIMLPKSLLSVVKSATLETKPESKYS

Tyrosines shown in bold are phosphorylated.

CHAPTER 3
CHARACTERIZATION OF THE SOCS-1 MIMETIC PEPTIDES

SOCS-1 Mimetics Recognize the Autophosphorylation Sites of Different JAKs

SOCS-1 has been reported to recognize and modulate the function of all four JAKs (Starr et al., 1997; Endo et al., 1997; Naka et al., 1997; Yoshimura et al., 2007). We were therefore interested in determining the ability of the SOCS-1 mimetics Tkip and SOCS1-KIR to bind to and modulate the kinase activity of JAK1, JAK2, JAK3, and TYK2. First, we plotted the hydropathic profiles of the JAK autophosphorylation sites. All four JAKs have similar protein sequences and therefore similar hydropathic profiles, suggesting similar structure (Figure 3-1a). Next, we synthesized peptides corresponding to the autophosphorylation sites of the four JAKs (Figure 3-1b) and compared them for binding to Tkip and SOCS1-KIR. As previously reported, both Tkip and SOCS1-KIR bound to the JAK2 autophosphorylation site (Figure 3-2a). Tkip and SOCS1-KIR also specifically bound to the autophosphorylation site peptide of TYK2 (Figure 3-2b), a JAK that plays a key role in mediation of type I IFN signaling (Leung et al., 1995). SOCS1-KIR, but not Tkip, bound to the JAK1 and JAK3 autophosphorylation site peptides (Figure 3-2c and d). This would suggest that the KIR region of SOCS-1 might be the key to SOCS-1 recognition of the JAKs.

Phenylalanines at positions 56 and 59 of SOCS-1 have previously been identified as critical for SOCS-1 binding to JAK2 and for its function (Yasukawa et al., 1999). Accordingly, the bindings of Figure 3-2 also include SOCS1-KIR with alanine substitutions at positions 56 and 59 (SOCS1-KIR2A), as well as Tkip with alanine substitutions at positions 8 and 11 (Tkip2A), which correspond to possible sites of homology with SOCS1-KIR (Flowers et al., 2004). SOCS1-KIR2A was unable to bind to

any of the JAKs, while Tkip2A showed reduced binding to JAK2 and TYK2 as compared to Tkip. Tkip2A and SOCS1-KIR2A did not bind to JAK1 or JAK3 autophosphorylation peptides.

SOCS-1 Mimetics are Not Cytotoxic

In order to assess possible toxicity of the SOCS-1 mimetic peptides, we incubated the peptides at various concentrations (3.7 to 100 μM) for 24 hours with murine L929 cells (Figure 3-3a) and splenocytes (Figure 3-3b). The assay was repeated for 72 hours with similar results. None of the peptides tested showed toxicity as assessed in a cytopathic assay. Thus, any biological effects of the test peptides are not due to toxicity.

SOCS-1 Mimetics Inhibit STAT Phosphorylation

As a functional correlate of the binding of Tkip and SOCS1-KIR to the autophosphorylation site peptides of the JAK kinases, we stimulated cells with IFNγ, IFNτ, or IL-10. The interaction of IFNγ with its receptor activates JAK1 and JAK2, which are responsible for the phosphorylation of STAT1 (Leaman et al., 1996). Both Tkip and SOCS1-KIR inhibited IFNγ-induced phosphorylation of STAT1 in L929 fibroblast cells (Figure 3-4a). Like other type I IFNs, IFNτ activates JAK1 and TYK2, which phosphorylate STAT1, STAT2, and STAT3 (Bazer et al., 1996). Tkip and SOCS1-KIR inhibited IFNτ-induced phosphorylation of STAT1 and STAT3 in L929 cells, and STAT2 in WISH cells (Figure 3-4b), while SOCS1-KIR2A and Tkip2A failed to inhibit the phosphorylation of STAT1 in response to IFNτ (Figure 3-4c), supporting the previous findings that they were unable to bind to the relevant JAKs. IL-10 also activates JAK1 and TYK2, which leads to phosphorylation of STAT1 and STAT3 (Finbloom and Winestock, 1995). Treatment of RAW264.7 macrophages with IL-10 resulted in

activation of STAT1 and STAT3, both of which were inhibited by Tkip and SOCS1-KIR (Figure 3-4d).

The Effect of SOCS-1 Mimetics on Macrophage Activation

SOCS-1 has been shown to inhibit LPS-induced activation of macrophages by blocking signal transduction events that occur via TLR4 (Mansell et al., 2006). TLR4 signaling involves activation of the adapter protein MAL by Bruton's tyrosine kinase (Btk), resulting in MAL-dependent p65 phosphorylation and the resultant activation of NF-κB (reviewed in Kobayashi et al., 2006). The induced SOCS-1 protein modulates this activation by binding to activated MAL, causing ubiquitination and then proteasomal degradation of MAL (Mansell et al., 2006). To determine if the SOCS-1 mimetic peptides could imitate the SOCS-1 inhibition of LPS-induced activation of these cells via TLR4, we treated murine RAW264.7 macrophages with Tkip and SOCS1-KIR, and determined the amount of nitric oxide produced. Both Tkip and SOCS1-KIR inhibited LPS-induced nitric oxide (NO) production (Figure 3-5). Based on a similar hydropathic profile of a tyrosine kinase phosphorylation site on MAL by Btk to that of pJAK2(1001–1013) (Figure 3-6a), we synthesized a MAL(82–94) peptide and determined its ability to bind to SOCS1-KIR. MAL(82–94) peptide showed a similar binding pattern to SOCS1-KIR as that of pJAK2(1001–1013) (Figure 3-6b). Another tyrosine kinase phosphorylation site on MAL, MAL(154–166), had a hydropathic profile different from pJAK2(1001–1013) (Figure 3-6a) and did not bind to SOCS1-KIR (Figure 3-6b).

We hypothesized that the binding of MAL(82–94) peptide to SOCS1-KIR reflected the mechanism of SOCS-1 recognition of MAL in LPS-treated cells. Accordingly, we treated RAW264.7 macrophages with SOCS1-KIR and MAL(82–94) to determine

whether the peptides could compete with endogenous proteins for binding to their targets. LPS treatment of cells in the presence of control peptide showed essentially complete degradation of MAL by 60 minutes, while LPS treatment in the presence of SOCS1-KIR completely blocked MAL degradation. Similarly, MAL(82–94) treatment also protected against MAL degradation (Figure 3-7). The inhibition of LPS activation of macrophages by SOCS1-KIR by binding to a critical functional site on MAL, yet blocking the SOCS-1-mediated degradation of MAL, suggests that inhibition of function can occur through the binding without the necessity for MAL degradation.

The Effects of SOCS1-KIR on SOCS-1$^{-/-}$ Mice

SOCS-1 is essential for survival, as evidenced by the fact that SOCS-1$^{-/-}$ mice die by three weeks of age due to severe inflammatory pathology (Starr et al., 1997; Naka et al., 1997). It was therefore of interest to determine the effects of SOCS1-KIR on the survival of SOCS-1$^{-/-}$ mice. We first established breeding pairs of SOCS-1$^{+/-}$ mice to generate progeny that would include SOCS-1$^{+/+}$, SOCS-1$^{+/-}$, and SOCS-1$^{-/-}$ pups. When the pups were born, we injected them with 10 μg per gram of body weight (measured immediately prior to injection) of SOCS1-KIR intraperitoneally (i.p.) every day. The pups were monitored daily for weight and signs of inflammation. The untreated SOCS-1$^{-/-}$ pups died by day 17, while the SOCS1-KIR treated mice survived until day 22 (Figure 3-8a).

SOCS-1$^{-/-}$ mice have stunted growth as compared to their wild-type littermates, so one of the parameters that we measured was weight change. The SOCS1-KIR treated knockout pups had weights that were similar to their wild-type littermates, up to a point (Figure 3-8b). This would suggest that SOCS1-KIR is able to at least partially remedy

the low body weight that is associated with the SOCS-$1^{-/-}$ phenotype. Further studies are being done in collaboration with Dr. Larkin's group, where the SOCS1-KIR peptide is being used in conjunction with adoptive transfer of $CD4^{+}CD25^{+}$ regulatory T cells to determine the survival outcome and reduction of inflammatory pathology of the SOCS-1 knockout mice.

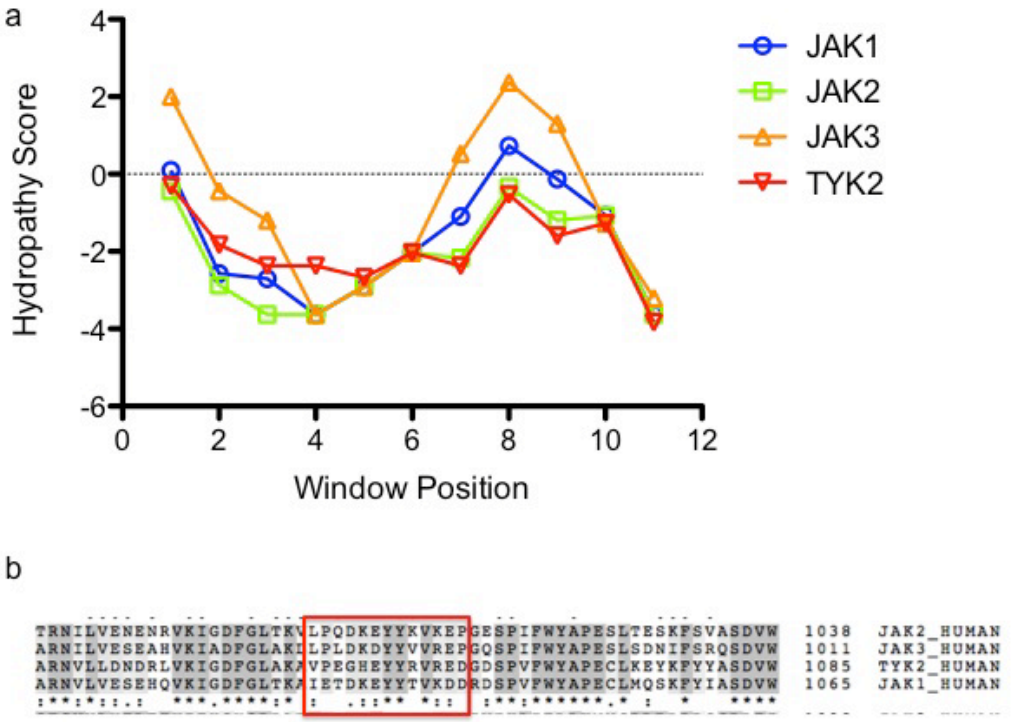

Figure 3-1. Autophosphorylation sites of the JAKs. **a.** A hydropathic plot of the autophosphorylation sites of the JAKs. **b.** A partial sequence alignment of the four JAKs, with the autophosphorylation sites outlined in red.

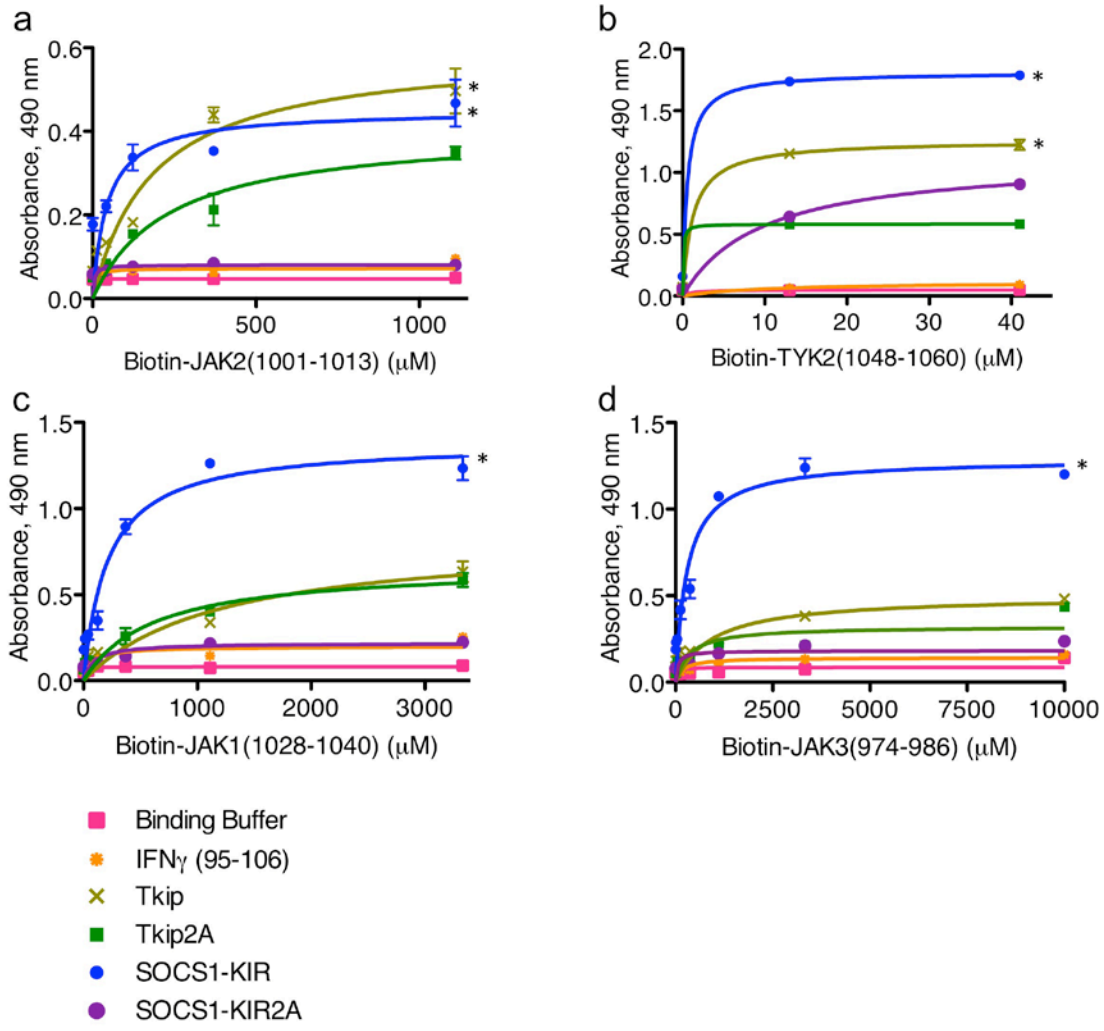

Figure 3-2. SOCS-1 mimetics bind to the autophosphorylation site peptides of the JAKs. Biotinylated pJAK2(1001–1013) (**a**), TYK2(1048–1060) (**b**), JAK1(1028–1040) (**c**), or JAK3(974–986) (**d**), at the indicated concentrations, were added in triplicate to a 96-well plate coated with binding buffer, IFNγ(95–106), SOCS1-KIR, SOCS1-KIR2A, Tkip, or Tkip2A, and binding assays were carried out as described in Chapter 2. Values represent the means (± SEM) of triplicate wells from three independent experiments. Stars (*) indicate statistically significant differences when compared to SOCS1-KIR2A or Tkip2A, as determined by the Mann-Whitney *U* test.

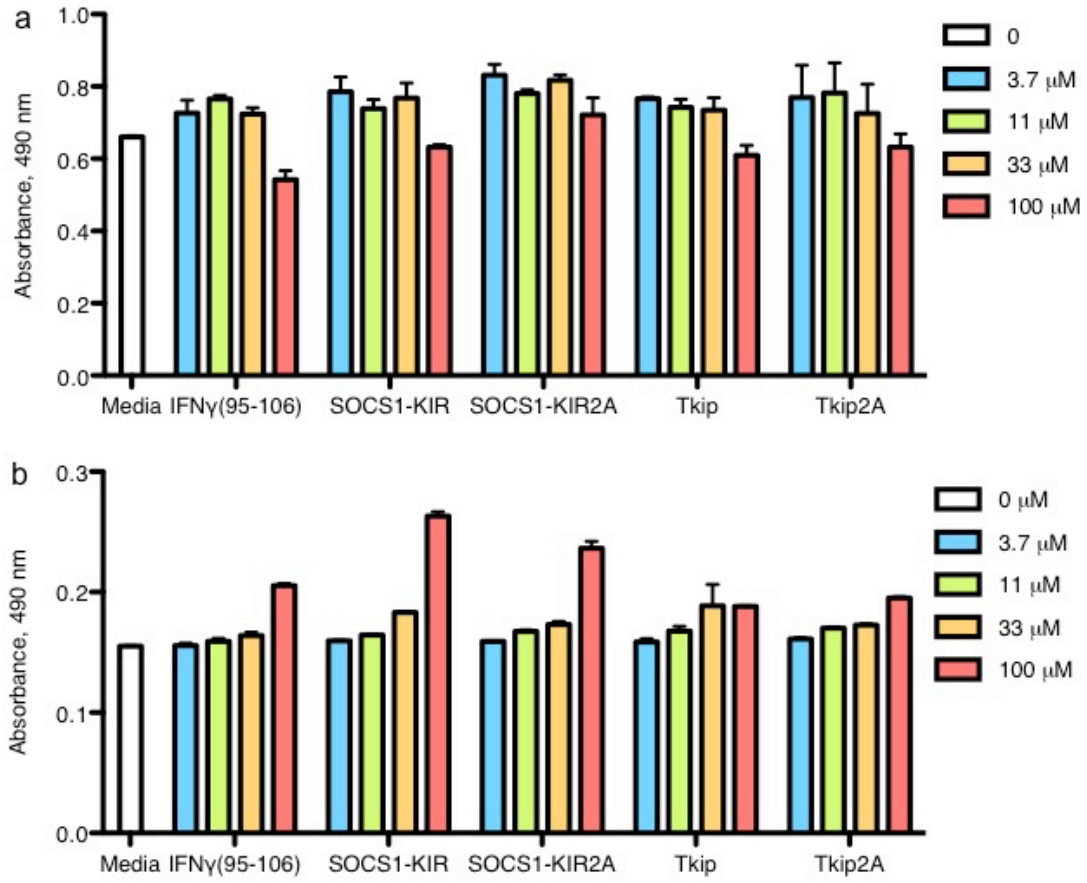

Figure 3-3. SOCS-1 mimetic peptides are not cytotoxic. L929 cells (**a**) or spleen cells isolated from healthy SJL/J mice (**b**) were seeded onto 96-well plates at 1×10^6 cells/ml. Peptides were added at various concentrations and the cells were incubated at 37°C at 5% CO_2. After 24 h, Solution Reagent was added and the cells were incubated for 2 h at 37°C, and the absorbance was measured at 490 nm. Values represent the means (± SEM) of triplicate wells from three independent experiments.

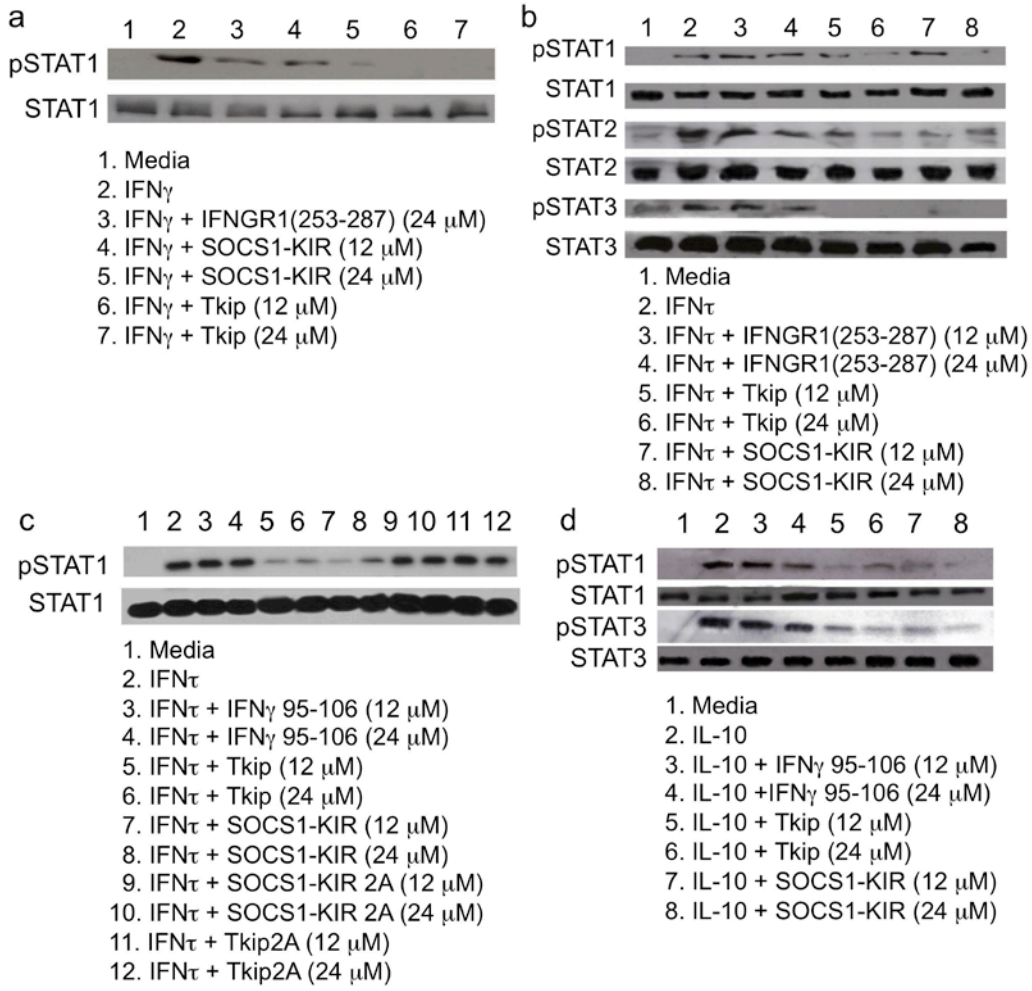

Figure 3-4. SOCS-1 mimetic peptides inhibit STAT phosphorylation. **a.** SOCS1-KIR and Tkip inhibit IFNγ-induced STAT1 activation. L929 cells were incubated with various peptides for 2 h at 37°C. Following a 2 h incubation in the presence or absence of IFNγ (4000 U/ml), the cells were washed and lysed. Whole cell extracts were resolved on 12% SDS-PAGE, transferred to nitrocellulose membranes, and the membranes were probed using pSTAT1 or STAT1 Ab. **b.** SOCS1-KIR and Tkip inhibit IFNτ-induced STAT1, STAT2, and STAT3 activation. L929 cells were treated as in part **a**, except 10,000 U/ml of IFNτ was used in lieu of IFNγ. Membranes were probed with Abs to pSTAT1, pSTAT2, pSTAT3, STAT1, STAT2, or STAT3. **c.** SOCS1-KIR2A and Tkip2A do not inhibit the phosphorylation of IFNτ-induced STAT1. L929 cells were treated as in part **b,** and membranes were probed with pSTAT1 or STAT1 Abs. **d.** SOCS1-KIR and Tkip inhibit IL-10-induced STAT1 and STAT3 activation. RAW264.7 cells were treated as in part **a**, except 10 ng/ml of IL-10 was used in lieu of IFNγ. Membranes were probed with Abs to pSTAT1, pSTAT3, STAT1, or STAT3. Data are representative of three independent experiments.

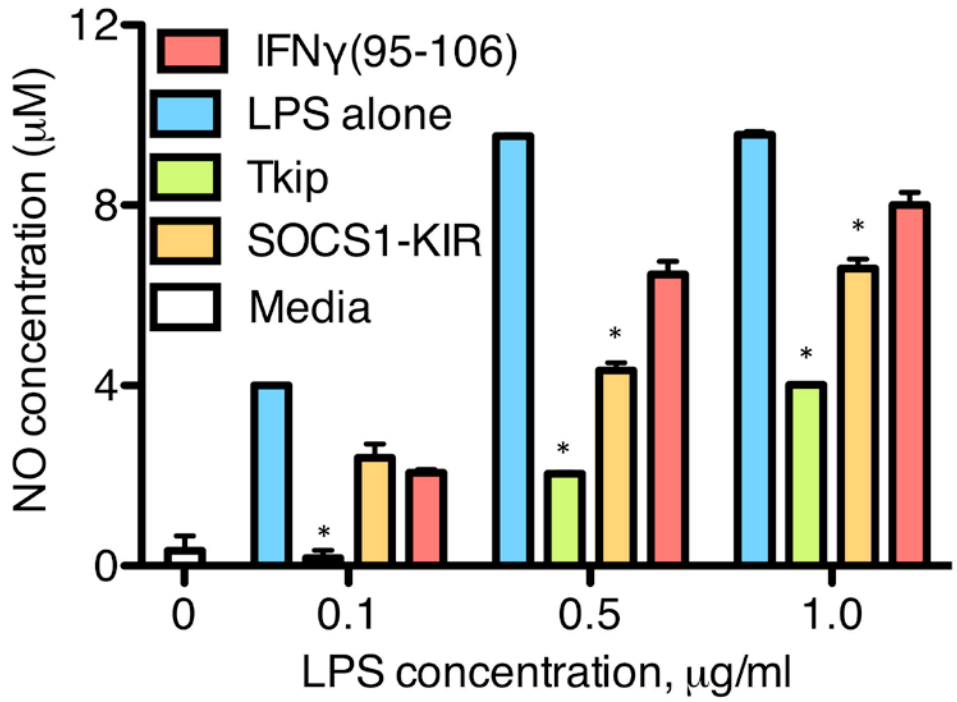

Figure 3-5. SOCS-1 mimetic peptides inhibit lipopolysaccharide (LPS) induced macrophage activation. RAW264.7 macrophages were incubated with varying concentrations of LPS alone or with various peptides at 24 μM for 48 h at 37°C. Culture supernatants were collected and nitric oxide concentration was determined using Griess reagent. Values represent the means (± SEM) of triplicate wells from three independent experiments. Stars (*) indicate statistically significant differences when compared to IFNγ(95–106)-treated cells, as determined by two-way ANOVA with Bonferroni post-tests.

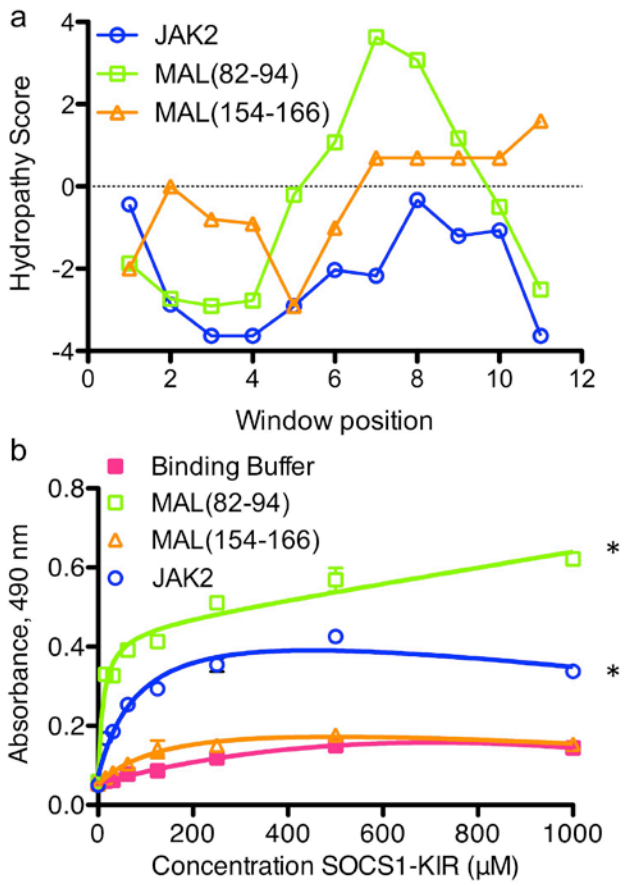

Figure 3-6. SOCS-1 mimetic peptides regulate TLR4 signaling. **a.** Hydropathic profiles of the JAK2 autophosphorylation site pJAK2(1001–1013) and the tyrosine phosphorylation site peptides of MAL. pJAK2(1001–1013) and MAL(82–94) show similar hydropathic profiles, while MAL(154–166) has a different hydropathic profile. **b.** SOCS1-KIR binds to MAL(82–94). Bindings were carried out as previously described with biotinylated MAL(82–94), MAL(154–166), and pJAK2(1001–1013). Values represent the means (± SEM) of triplicate wells from three independent experiments. Stars (*) indicate statistically significant differences ($p < 0.05$) when compared to binding buffer, as determined by the Mann-Whitney *U* test.

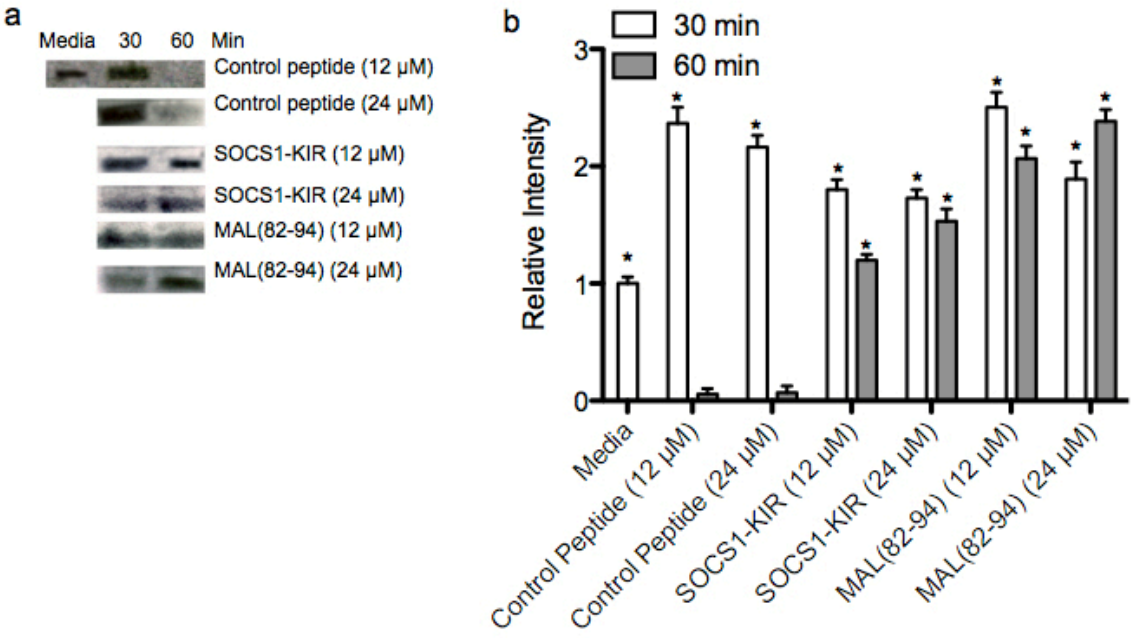

Figure 3-7. SOCS1-KIR inhibits MyD88 adapter like (MAL) protein degradation. a. RAW264.7 macrophages were seeded onto 6-well plates at 2.5 x 10^6 cells/ml and treated with LPS (1 μg/ml) in the presence or absence of SOCS1-KIR, MAL(82–94) or IFNγ(95–106) (control peptide) for the indicated times. Westerns were performed as previously described and probed with Ab to MAL. The media lane is a negative control for all treatments, which were carried out in one experiment. Data are representative of three independent experiments. Densitometry is presented in part b. Values represent the means (± SEM) of western blot bands from three independent experiments. Stars (*) indicate statistically significant differences when compared to 60 min Control Peptide (12 μM) treated cells, as determined by two-way ANOVA with Bonferroni post-tests.

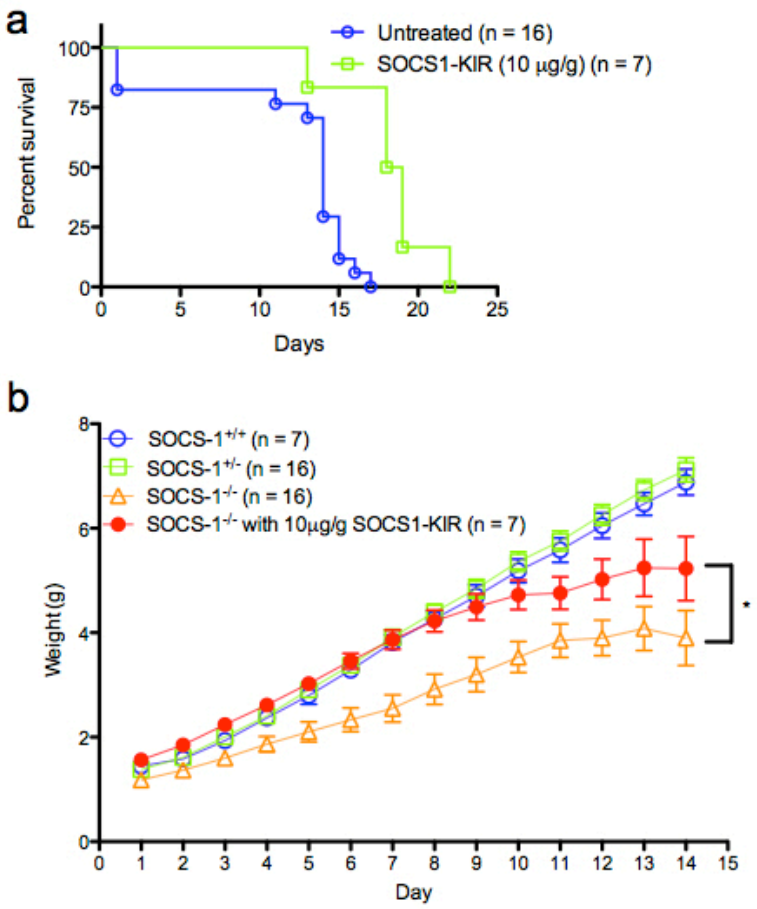

Figure 3-8. SOCS1-KIR prolongs survival of SOCS-$1^{-/-}$ mice. Pups were injected daily with 10 μg per gram body weight of SOCS1-KIR, i.p., and monitored for survival (**a**) and weight change (**b**). There were statistically significant differences between the survival of treated and untreated SOCS-$1^{-/-}$ mice, as determined by the log-rank test. There were statistically significant differences between weights of untreated and treated SOCS-$1^{-/-}$ mice, as determined by a two-way ANOVA.

CHAPTER 4
CHARACTERIZATION OF THE SOCS-1 ANTAGONIST PEPTIDE

The SOCS-1 Antagonistic Activity of pJAK2(1001-1013)

As previously mentioned, SOCS1-KIR can bind to the autophosphorylation site peptide of JAK2, pJAK2(1001–1013) (Figure 3-2). This raised the possibility that pJAK2(1001–1013) can inhibit the function of endogenous SOCS-1 and thus enhance the various cytokine activities that are mediated by JAK2. Previous studies done in our lab have shown that the pJAK2(1001–1013) peptide can enhance the antiviral activity of IFNγ, increase STAT3 activation in IL-6-treated cells that overexpress SOCS-1, enhance the GAS promoter activity of IFNγ, and enhance antigen-specific splenocyte proliferation (Waiboci et al., 2007). In addition, it was shown that SOCS-1 protein competes with SOCS1-KIR for binding to pJAK2(1001–1013) (Waiboci et al., 2007). This suggests that pJAK2(1001–1013) acts as a SOCS-1 antagonist.

The SOCS-1 Antagonist Increases STAT1 Phosphorylation

To determine if the SOCS-1 antagonist had any effect on STAT1 activation, we treated L929 cells with pJAK2(1001–1013) and JAK2(1001–1013)2A (alanines substituted for tyrosines 1007 and 1008), and probed for phosphorylated STAT1 (Figure 4-1). Treatment with pJAK2(1001–1013) at 25 μM increased pSTAT1 levels by more than two-fold over untreated cells, while JAK2(1001–1013)2A had minimal effects.

The SOCS-1 Antagonist Increases Endogenous IFNβ Levels

For efficient induction of an antiviral state, cells contain low levels of constitutive IFNβ (Taniguchi and Takaoka, 2002). A subtle increase in this low level of IFNβ plays an important role in a positive feedback loop to increase type I IFN production and induction of a potent antiviral state in cells (Takaoka et al., 2000). To determine if

pJAK2(1001–1013) affected the level of constitutive IFNβ, we treated L929 fibroblasts with pJAK2(1001–1013) and JAK2(1001–1013)2A. Cells treated with pJAK2(1001–1013) for 30 or 60 minutes showed an increase in IFNβ as determined by Western blot, while JAK2(1001–1013)2A had little or no effect on IFNβ levels (Figure 4-2a). In comparison, IFNα levels were not altered in the same cells (Figure 4-2b). Western blots showed a decline in SOCS-1 protein levels in pJAK2(1001–1013)-treated cells, while JAK2(1001–1013)2A had no discernable effect on SOCS-1 protein levels (Figure 4-2c). This decrease in SOCS-1 levels corresponded to the increase in IFNβ levels in the cells, and this would suggest that pJAK2(1001–1013) played a role in SOCS-1 degradation.

To confirm the increase in IFNβ protein levels in L929 cells treated with pJAK2(1001–1013), we determined the intracellular IFNβ levels by ELISA. The cells treated with pJAK2(1001–1013) had an approximately two-fold increase in IFNβ levels as compared to untreated cells (Figure 4-2d). JAK2(1001–1013)2A-treated cells had IFNβ levels comparable to those of untreated cells.

The Effects of the SOCS-1 Antagonist on Macrophage Activation

SOCS-1 negatively regulates TLR signaling at several stages, including signaling by type I IFNs and by the transcription factor NF-κB. Since the SOCS-1 mimetic peptides are able to inhibit macrophage activation, we were interested to see if the SOCS-1 antagonist peptide would increase macrophage activation. We treated RAW264.7 macrophages with pJAK2(1001–1013) and JAK2(1001–1013)2A and measured the amount of NO produced upon LPS stimulation. The pJAK2(1001–1013)

treated cells produced an approximately five-fold increase in NO, while the JAK2(1001–1013)2A peptide had a minimal effect (Figure 4-3a).

We also examined the effect of pJAK2(1001–1013) on TLR3 activation. TLR3 recognizes double-stranded RNA (dsRNA) and the synthetic dsRNA analog polyriboinosinic:polyribocytidylic acid (poly I:C) and induces type I IFN (Matsumoto and Seya, 2008). TLR3 plays an important role in the antiviral response to viruses that have a dsRNA stage in their life cycle, including HSV-1, influenza virus, cytomegalovirus, and respiratory syncytial virus (Vercammen et al., 2008). We treated RAW264.7 macrophages with pJAK2(1001–1013) and JAK2(1001–1013)2A, and determined the effects on poly I:C-induced NO production. The pJAK2(1001–1013) treatment increased NO production over 20-fold as compared to poly I:C alone, while JAK2(1001–1013)2A had a negligible effect (Figure 4-3b).

The SOCS-1 Antagonist is Not Cytotoxic

In order to assess possible toxicity of the SOCS-1 antagonist peptide, we incubated pJAK2(1001–1013) and JAK2(1001–1013)2A at various concentrations (3.7 to 25 μM) with murine L929 cells (Figure 4-4). None of the peptides tested showed significant toxicity as assessed in a cytopathic assay. Thus, any biological effects of the test peptides are not due to toxicity.

The SOCS-1 Antagonist Possesses Antiviral Activity Against a Picornavirus

Encephalomyocarditis virus (EMCV) is a small single-stranded RNA picornavirus of the plus strand orientation with a wide host range. EMCV infection can cause myocarditis leading to arrhythmias, heart failure, and death (Robinson et al., 2009). During cardiac transplantation and valve replacement, infection by EMCV has been

implicated in the development of cardiomyopathy, which makes the development of effective therapies against this virus particularly important (Yajima and Knowlton, 2009).

In mice, EMCV infection is lethal, but is quite susceptible to IFNγ or an IFNγ mimetic (IFNγ(95–132)) treatment at early stages of infection (Mujtaba et al., 2006). The IFNγ mimetic is a small peptide that corresponds to the C-terminus of IFNγ and functions intracellularly. It has been shown to be an effective treatment against vaccinia virus (Ahmed et al., 2007) and herpes simplex virus 1 (HSV-1) (Frey et al., 2009).

We reasoned that the SOCS-1 antagonist peptide would either induce or enhance an antiviral state by limiting the ability of SOCS-1 to modulate constitutive or added IFN antiviral activity. We have previously shown that pJAK2(1001–1013) antagonized the effect of SOCS-1 in HSV-1 infected keratinocytes (Frey et al., 2009). It reduced HSV-1 titers by two-fold as compared to untreated cells (Table 4-1) (Frey et al., 2009). We were therefore interested to see whether pJAK2(1001–1013) would have an antiviral effect against EMCV.

We treated L929 fibroblasts with pJAK2(1001–1013), JAK2(1001–1013)2A, IFNγ, the control peptide IFNγ(95–125), or IFNγ(95–132) prior to infection with 200 plaque forming units (pfu) of EMCV. Both IFNγ and IFNγ(95–132) as well as pJAK2(1001–1013) inhibited EMCV infection (Figure 4-5a). Specifically, pJAK2(1001–1013) and IFNγ reduced cytopathic effects (CPE) by approximately 50%, while IFNγ(95–132) was completely protective. The JAK2(1001–1013)2A peptide was only about 7% protective.

Synergy between pJAK2(1001–1013) and IFNγ(95–132) was observed in treatments using suboptimal concentrations of both peptides, where 2 μM of pJAK2(1001–1013) and 5 μM of IFNγ(95–132) combined completely protected L929

cells against EMCV infection, while separately the peptides at these concentrations showed 20% or less protection (Figure 4-5b).

The increase of IFNβ in cells treated with pJAK2(1001–1013) raises the possibility that this IFN exerts its effects intracellularly and thus does not need to be secreted for subsequent interaction with the extracellular domain of the type I IFN receptor. To address this, we treated L929 cells with pJAK2(1001–1013) in the presence or absence of neutralizing antibodies to IFNβ prior to infection with 200 pfu of EMCV. Complete protection by pJAK2(1001–1013) was reduced to approximately 60% in the presence of a saturating level of anti-IFNβ antibody (Figure 4-5c). JAK2(1001–1013)2A was not protective. These data suggest that some of the increased IFNβ exerted its effects intracellularly.

Based on the antiviral effects of pJAK2(1001–1013) in tissue culture, we tested the therapeutic effects of the antagonist in a mouse model of lethal EMCV infection. C57BL/6 mice were treated i.p. with 50, 100, or 200 μg of pJAK2(1001–1013) or 200 μg of JAK2(1001–1013)2A every day beginning 2 days prior to challenge with 50 pfu of EMCV per mouse, and the mice were monitored daily for signs of infection. Mice treated with JAK2(1001–1013)2A all died by day 5 after EMCV challenge (Figure 4-6a). In contrast, mice treated with 100 and 200 μg pJAK2(1001–1013) showed 80% and 60% survival, respectively. Treatment with 50 μg of pJAK2(1001–1013) resulted in 20% survival.

To assess synergistic effects between pJAK2(1001–1013) and IFNγ(95–132), mice were treated with suboptimal doses of both peptides every day beginning 2 days prior to challenge with 50 pfu of EMCV. Treatment with pJAK2(1001–1013) at 10 μg and

IFNγ(95–132) at 2 μg resulted in 80% survival of infected mice, while treatment with pJAK2(1001–1013) alone resulted in 40% survival, and IFNγ(95–132) alone resulted in 60% survival (Figure 4-6b).

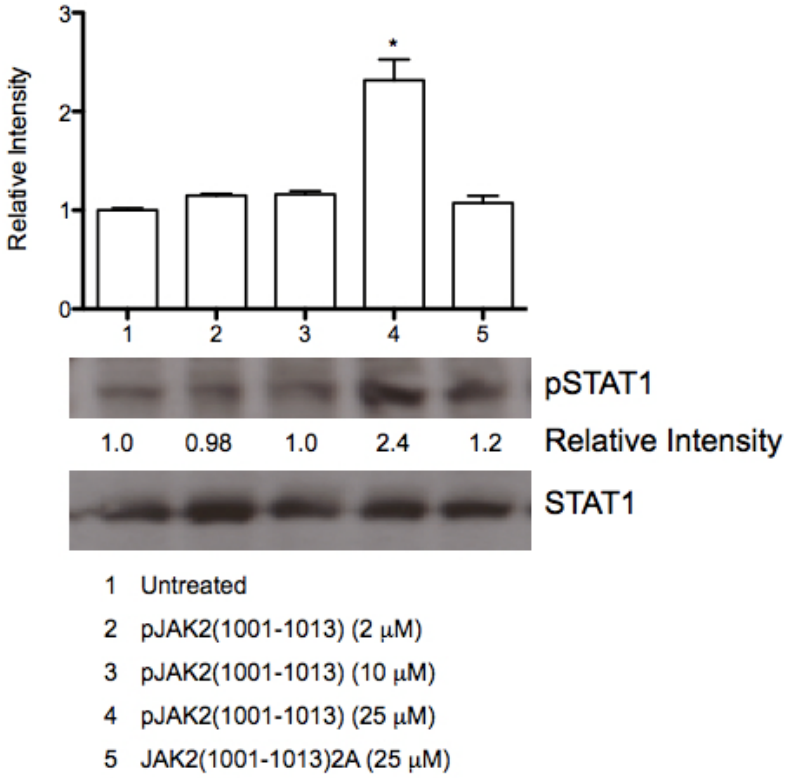

Figure 4-1. The SOCS-1 antagonist peptide increases STAT1 phosphorylation. L929 cells were seeded onto a 6-well plate at 1 x 10^6 cells/ml, grown to confluency, and incubated with pJAK2(1001–1013) or JAK2(1001–1013)2A for 1 h. Cells were lysed and whole cell lysates were resolved by 12% SDS-PAGE, proteins were transferred onto nitrocellulose membrane, and the membranes were probed with pSTAT1 and STAT1 antibodies. Data are representative of three independent experiments. Values represent the means (± SEM) of bands from three independent experiments. Stars (*) indicate statistically significant differences when compared to untreated cells, as determined by one-way ANOVA with Bonferroni post-tests.

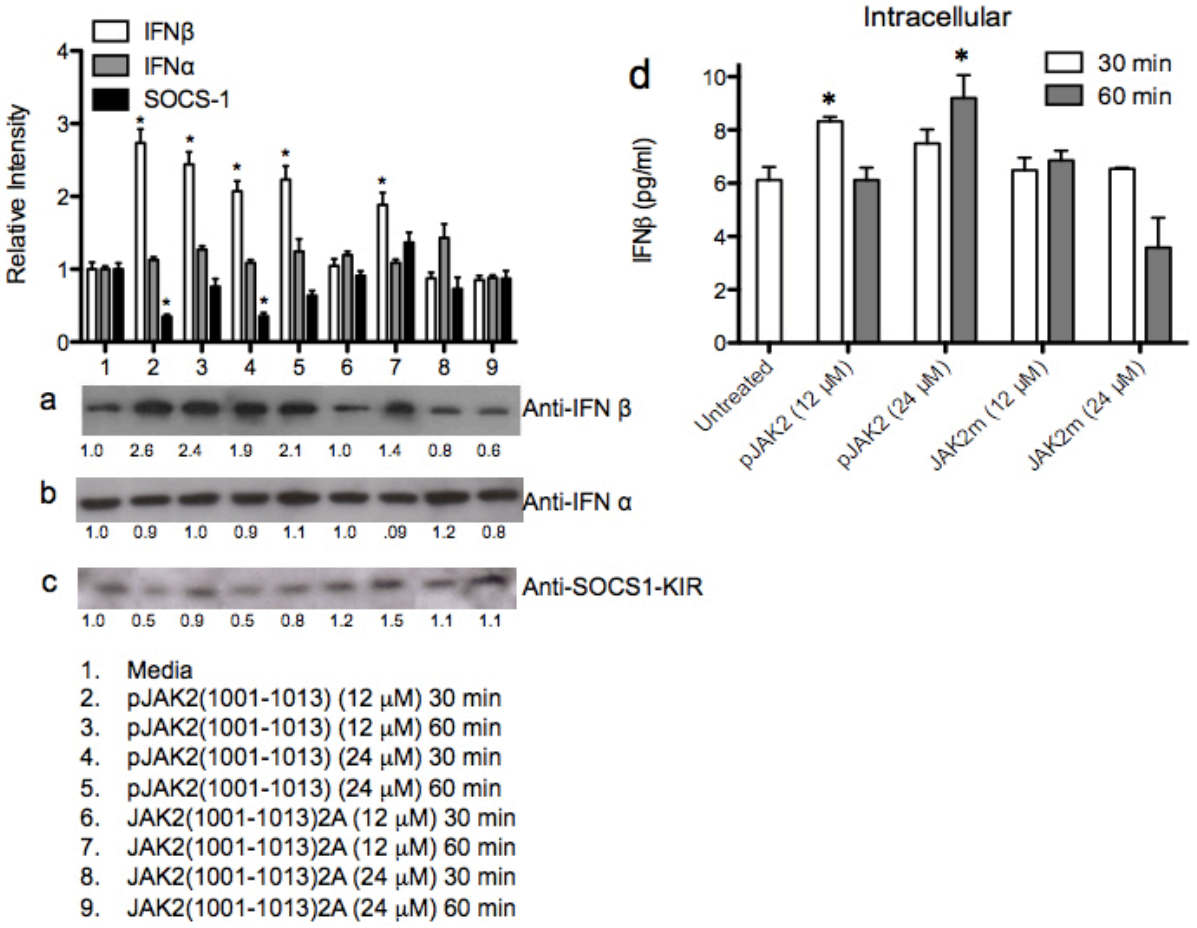

Figure 4-2. The SOCS-1 antagonist increases endogenous interferon β levels. **a-c.** L929 cells were seeded onto 6-well plates at 1 x 10^6 cells/ml and grown to confluence. The cells were incubated with pJAK2(1001–1013) and JAK2(1001–1013)2A (JAK2m) for 30 or 60 minutes at 37°C. The cells were lysed and immunoblotted as previously described for IFNβ (**a**), IFNα (**b**), or SOCS-1 (**c**). Relative intensities are shown in numbers below each blot, and in a graph above all blots. Values represent the means (± SEM) of bands from three independent experiments. Stars (*) indicate statistically significant differences when compared to untreated cells, as determined by one-way ANOVA with Bonferroni post-tests. **d.** Intracellular IFNβ levels were determined for the cell lysates with an IFNβ ELISA kit, following manufacturer's instructions. Values represent the means (± SEM) of triplicate wells from three independent experiments. Stars (*) indicate statistically significant differences when compared to untreated cells, as determined by two-way ANOVA with Bonferroni post-tests.

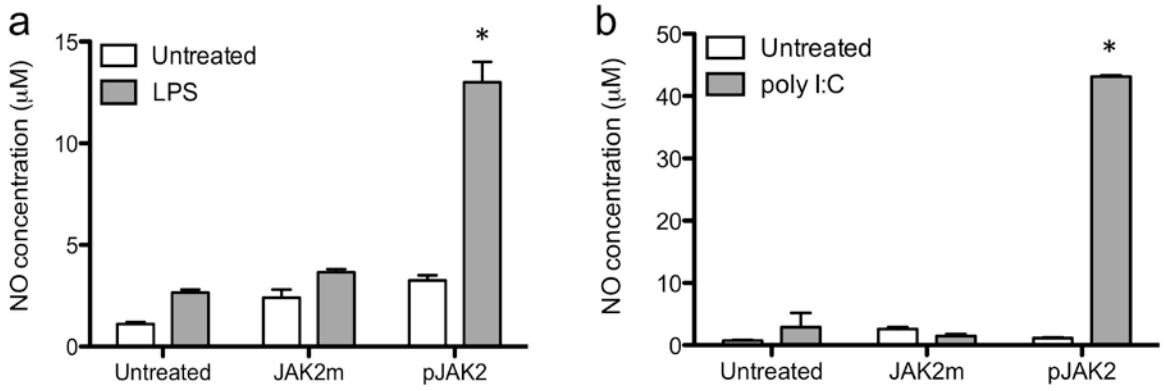

Figure 4-3. The SOCS-1 antagonist increases macrophage activation. pJAK2(1001–1013) increases LPS-induced macrophage activation. RAW264.7 cells were seeded onto 24-well plates at 5 x 10^6 cells/ml and grown overnight. They were incubated with pJAK2(1001–1013) (pJAK2) or JAK2(1001–1013)2A (JAK2m) at 24 μM for 4 h, after which 2 μg/ml of LPS (**a**) or 0.1 μg/ml of poly I:C (**b**) was added and the cells were incubated for 48 h at 37°C. Supernatants were collected and nitric oxide concentration was determined using Griess reagent. Values represent the means (± SEM) of triplicate wells from three independent experiments. Stars (*) indicate statistically significant differences when compared to untreated cells, as determined by two-way ANOVA with Bonferroni post-tests.

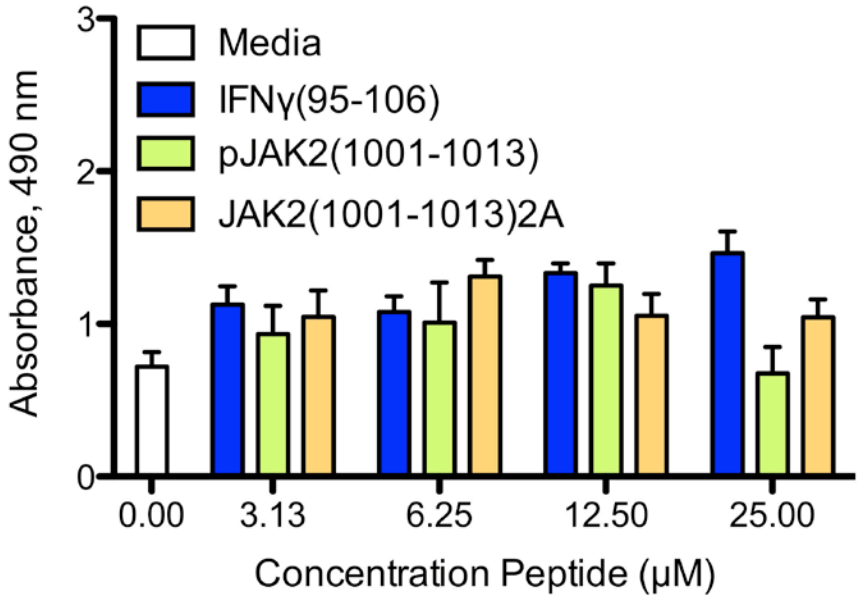

Figure 4-4. The SOCS-1 antagonist is not cytotoxic. L929 cells were seeded onto 96-well plates at 1 x 10^6 cells/ml. Peptides were added at various concentrations and the cells were incubated at 37°C at 5% CO_2. After 24 h, Solution Reagent was added and the cells were incubated for 2 h at 37°C, and the absorbance was measured at 490 nm. Values represent the means (± SEM) of triplicate wells from three independent experiments.

Table 4-1. Yield reduction of herpes simplex virus 1.

Treatment	Virus Yield (pfu/ml)	Fold Reduction
Untreated	7×10^7	
pJAK2(1001–1013) (25 μM)	4×10^7	2
IFNγ(95–132) (25 μM)	5×10^6	14
IFNγ (95–132) (50 μM)	9×10^5	77

Frey et al., 2009.

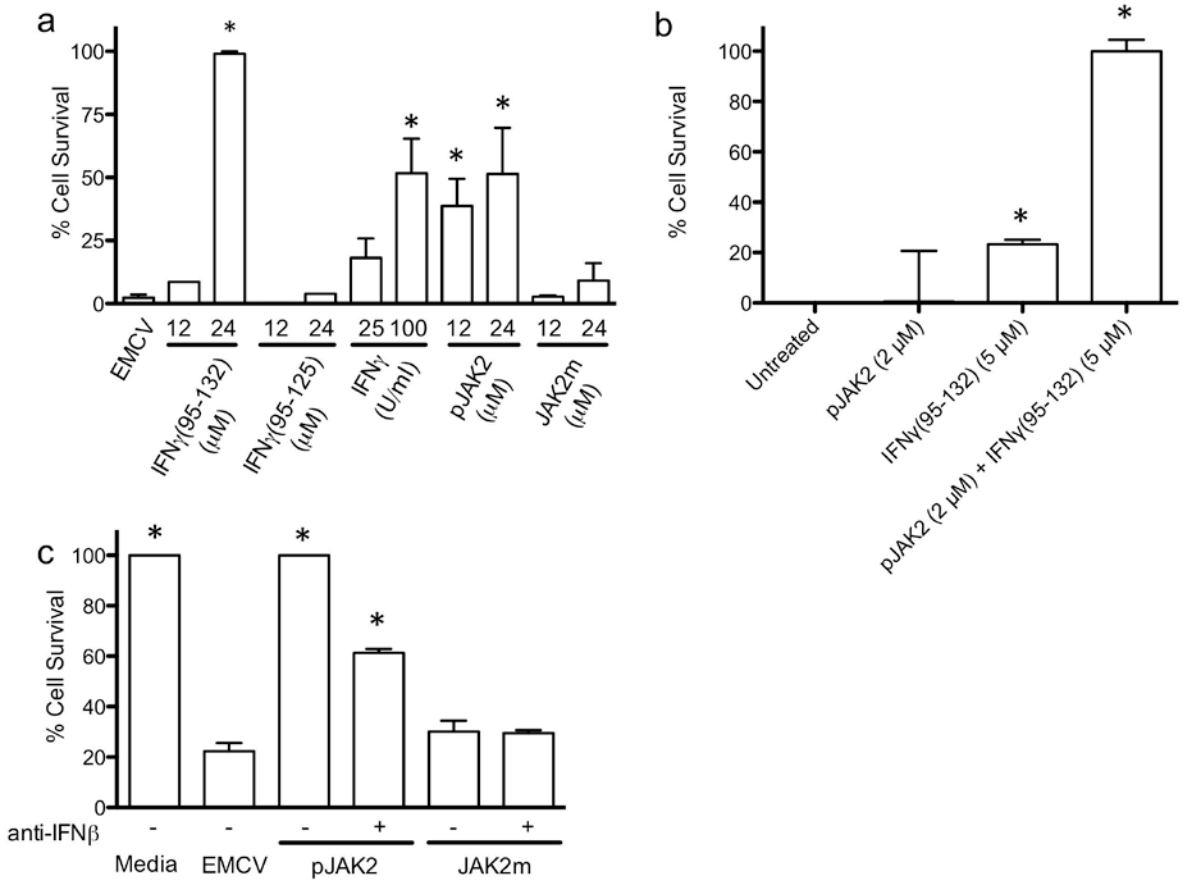

Figure 4-5. The SOCS-1 antagonist has antiviral activity against encephalomyocarditis virus (EMCV). **a.** L929 cells were treated with IFNγ, pJAK2(1001–1013) (pJAK2), JAK2(1001–1013)2A (JAK2m), IFNγ(95–125), or IFNγ(95–132) for 2 h, after which 200 pfu of EMCV were added. After 1 h, virus was removed and fresh media was added followed by incubation for 24 h at 37°C. Cells were stained with crystal violet and the plates were scanned and analyzed using ImageJ 1.29 software. **b.** Synergy between pJAK2(1001-1013) and IFNγ(95–132) in inhibition of EMCV. L929 cells were treated as in part a, and pJAK2(1001–1013) at 2 μM and IFNγ(95–132) at 5 μM were incubated together as well. **c.** L929 cells were incubated with the peptides (24 μM) in the presence or absence of 500 U/ml of neutralizing antibody to IFNβ for 2 h, after which the cells were infected with EMCV and processed as in part **a**. Values represent the means (± SEM) of triplicate wells from three independent experiments. Stars (*) indicate statistically significant differences when compared to EMCV-infected cells, as determined by two-way ANOVA with Bonferroni post-tests.

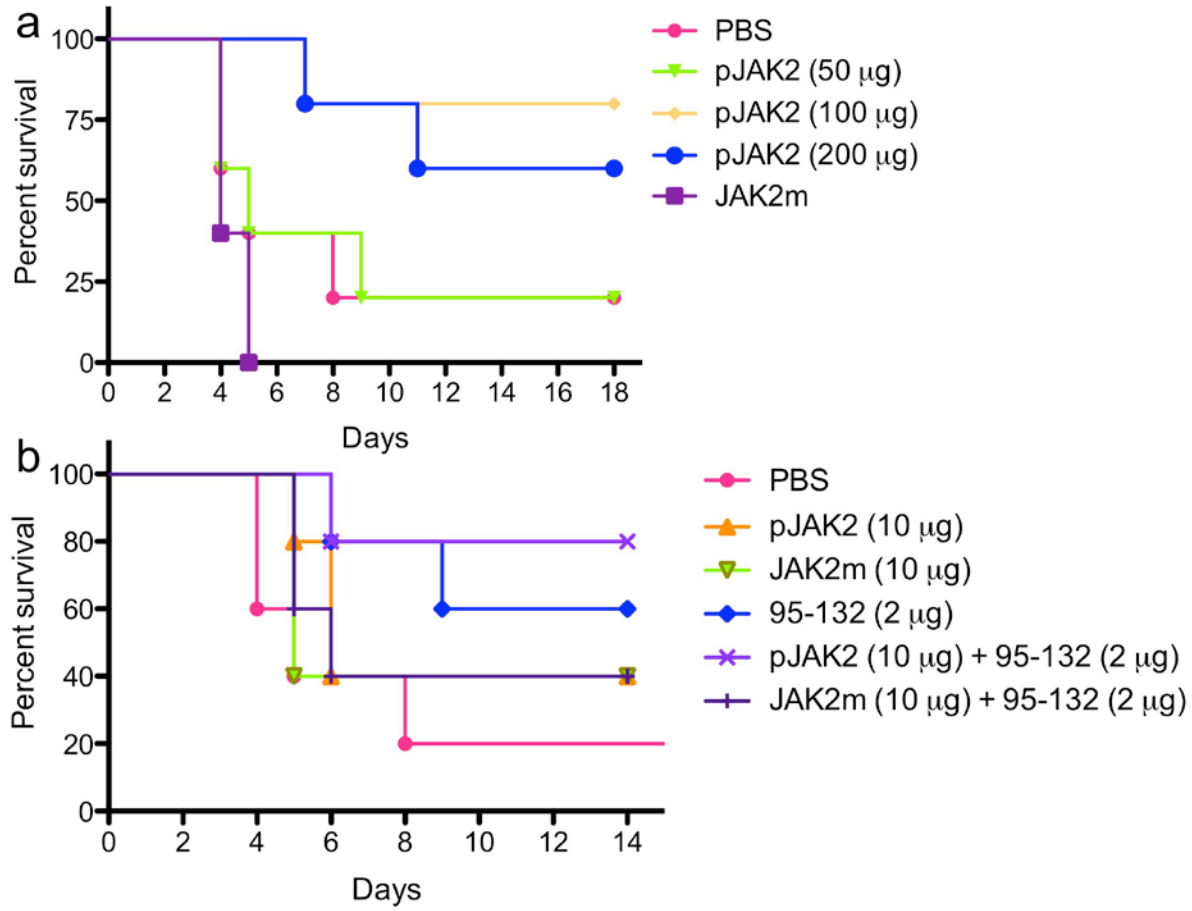

Figure 4-6. The SOCS-1 antagonist protects mice from lethal EMCV infection. **a.** C57BL/6 mice (n = 5 per group) were injected daily i.p. beginning at day -2 with pJAK2(1001–1013) (pJAK2) at 50, 100, and 200 μg and JAK2(1001–1013)2A (JAK2m) at 200 μg. On day 0, 50 pfu of EMCV per mouse were injected i.p. Survival data are presented as Kaplan-Meier plots. Survival curves were not found to be significantly different when compared to the PBS treated group, as determined by the log-rank statistical test. **b.** Synergy in protection of mice infected with EMCV as for part **a**, using suboptimal levels of pJAK2(1001–1013) (pJAK2) (10 μg) and IFNγ(95–132) (95–132) (2 μg). Survival data are presented as Kaplan-Meier plots. Survival curves were not found to be significantly different when compared to the PBS treated group, as determined by the log-rank statistical test.

CHAPTER 5
DISCUSSION

Knocking out the SOCS-1 gene in mice results in neonatal death. The mice appear to be normal at birth, but exhibit stunted growth and die at approximately 3 weeks of age (Starr et al., 1997). The primary cause of the pathology that leads to death is unregulated or inadequate regulation of IFNγ activity, since SOCS-1 knockout mice that are deficient in IFNγ or IFNγ receptor do not die as neonates (Alexander et al., 1999). How SOCS-1 keeps the IFNγ system under control is complex. Structurally, optimal recognition of JAK2 by SOCS-1 involves a 12-residue KIR region, a 12-residue extended SH2 sequence (ESS), and a longer SH2 domain (Yasukawa et al., 1999). This study has shown that the KIR can recognize the autophosphorylation site of JAK2 independently of the ESS and SH2 regions, using the SOCS1-KIR peptide. SOCS1-KIR can also recognize the autophosphorylation site of the other JAKs, and it can block the phosphorylation of various STATs associated with type I and II IFN, as well as IL-10, signaling.

The ability of the KIR region to inhibit IFNγ and other cytokine functions is of interest in assessing other functional domains of SOCS-1, particularly the SOCS box domain. SOCS-1, as well as the other seven SOCS proteins, contains a homology domain called the SOCS box that makes up the C-terminus of the protein (Yasukawa et al., 1999). The conserved SOCS box domain binds to elongins B and C, which form part of an E3 ubiquitin ligase complex that ubiquitinates proteins and targets them for proteasomal degradation (Kamura et al., 2004). Deleting only this domain from the SOCS-1 gene and comparing the mouse phenotype to that of mice lacking the entire SOCS-1 protein determined the role of the SOCS box in SOCS-1 function (Zhang et

al., 2001). The SOCS box knockout mice, although possessing increased responsiveness to IFNγ, did not die by 3 weeks as the SOCS-$1^{-/-}$ mice do. Rather, these mice had approximately 50% survival at 50 days, and approximately 20% survival as late as 90 days. Prolonged activation of STAT1 in hepatocytes was intermediate between that of wild-type and SOCS-$1^{-/-}$ mice. This suggests that the protective effect of SOCS-1 in wild-type mice against unregulated IFNγ appears to be due to both KIR/SH2 binding to JAK2 and proteasomal degradation of JAK2 via activity of the SOCS box.

We have shown in this study that both the SOCS1-KIR and Tkip peptides bound to JAK2 and TYK2, while only SOCS1-KIR bound to JAK1 and JAK3. This is consistent with SOCS-1 recognition of all of the JAKs (reviewed in O'Sullivan et al., 2007). This study has also shown that the SOCS-1 mimetic peptides inhibit type I IFN-induced activation of STAT1, STAT2, and STAT3. IL-10 treatment of macrophages resulted in activation of STAT1 and STAT3, which were also inhibited by the SOCS-1 mimetic peptides. Additionally, the SOCS-1 mimetic peptides blocked IFNγ-induced STAT1 phosphorylation, as previously shown (Waiboci et al., 2007). The demonstration here that SOCS1-KIR binds to all of the JAKs provides a direct correlate to SOCS-1 induction by and regulation of numerous cytokines, growth factors, and hormones that use the JAK/STAT signaling pathway.

Using alanine substitutions for essential phenylalanines at positions 56 and 59 of SOCS-1, we showed that SOCS1-KIR2A failed to recognize all four JAKs and did not block IFNτ-induced activation of STAT1. Alanine substitutions at potential phenylalanine homology sites 8 and 11 of Tkip, Tkip2A, resulted in reduced binding to JAK2 and TYK2 and loss of ability to inhibit IFNτ-induced activation of STAT1. This would suggest that

there is specificity involved in the recognition of the autophosphorylation sites of the JAKs by the SOCS-1 mimetic peptides, which is consistent with previously identified critical phenylalanine residues in KIR (Yasukawa et al., 1999).

The inhibition of LPS-induced activation of macrophages by the SOCS-1 mimetic peptides demonstrates that they, like SOCS-1, can regulate TLR4 function (Mansell et al., 2006). SOCS-1 regulates TLR4 at multiple sites in macrophage signaling, including MAL, p65 of NF-κB, an IRAK tyrosine kinase, or induced IFN autocrine activation of JAKs (Kobayashi et al., 2006).

MAL is one of the adapter proteins that are involved in TLR4 signaling (Fitzgerald et al., 2001). Its activation leads in turn to activation of the transcription factor NF-κB. Enhanced binding of SOCS-1 to activated MAL results in its proteasomal degradation (Mansell et al., 2006). We identified a binding site on MAL for the KIR region of SOCS-1, which is consistent with SOCS1-KIR peptide inhibition of TLR4 signaling. We also showed that SOCS1-KIR competes with endogenous SOCS-1 protein for binding to MAL, and that SOCS1-KIR prevents the proteasomal degradation of MAL while inhibiting TLR4 signaling. This would suggest that SOCS1-KIR is able to block MAL function without the need for proteasomal degradation, since SOCS1-KIR lacks the SOCS box.

SOCS-1 acts intracellularly in the cells in which it is induced and this is most likely the key to the selective aspects of its regulation of various functions. The use of the SOCS-1 mimetic peptides as therapeutics for immunological or other disorders presents a potential challenge in inhibition of inflammatory cytokines such as IFNγ, while minimally affecting anti-inflammatory cytokines such as IL-10, since the mimetics are

able to inhibit the actions of both. We have previously shown that Tkip has therapeutic effectiveness in the experimental allergic encephalomyelitis (EAE) model of multiple sclerosis under a given protocol of dosage and time of Tkip administration (Mujtaba et al., 2005). This demonstrates that these peptides should have therapeutic value in SOCS-1 based treatment of immunological diseases. Studies currently under way in our lab have shown that SOCS1-KIR can also be used as a therapeutic for EAE, as treatment with SOCS1-KIR lowered disease incidence and the resulting pathology to levels that are similar to those of naive mice (unpublished data). In addition to treatment of autoimmune diseases, SOCS1-KIR is able to prolong survival of SOCS-$1^{-/-}$ mice and reduce the associated inflammation (unpublished data).

Regulation of the SOCS-1 modulatory arm of the immune response provides an approach to enhancement of the response to infectious agents as well as to “weak” antigens that are the target of tumor vaccine studies. In this regard, the regulatory role of SOCS-1 extends to dendritic cells (DCs) and antigen presentation. Recently, it was shown that knockdown of DC SOCS-1 by siRNA led to more effective cancer vaccination (Shen et al., 2004). Specifically, presentation of murine melanocyte differentiation antigen tyrosine-related protein 2 (TRP2) by DCs transfected with SOCS-1 siRNA protected C57BL/6 mice against the well-established B16 melanoma tumor. Protection was not observed in DC vaccination where the siRNA was disrupted by GFP. The enhanced anti-tumor immunity was accompanied by enhanced TRP2-specific cytotoxic T cells (CTLs) in protected mice as assessed by IFNγ ELISPOT and CTL responses. The authors concluded that regulation of antigen presentation by suppression of DC SOCS-1 showed promise for more effective tumor vaccines. The

SOCS-1 siRNA treatment also enhanced HIV-1 envelope specific $CD8^+$ CTL responses, which suggests that suppression of SOCS-1 in DCs is of potentially general value for immune enhancement against AIDS (Song et al., 2006). These observations are related to the observation that SOCS-$1^{-/-}$ mice are more resistant to viral infection than their wild-type counterparts, due to enhanced type I IFN activity involving the IFNAR1 subunit (Zimmerer et al., 2007).

The development of the SOCS-1 antagonist pJAK2(1001–1013) was based on the observation that the KIR region of SOCS-1 binds directly to the autophosphorylation site peptide of JAK2, raising the possibility that the peptide could function as an antagonist of SOCS-1 (Waiboci et al., 2007). It was shown that pJAK2(1001–1013) enhanced suboptimal IFNγ activity, reversed the SOCS-1-induced reduced activation of STAT3 by IL-6 treatment, enhanced IFNγ activation of the luciferase reporter gene via the GAS promoter element, and enhanced antigen-specific splenocyte proliferation (Waiboci et al., 2007).

The antiviral effects of the SOCS-1 antagonist appear to operate through direct effects on the cell as well as by indirect effects in mice by enhancement of both the cellular and humoral arms of the immune system. TLRs are key players in both the innate and adaptive arms of host defense. With respect to TLR3 and virus immunity, treatment of the macrophage cell line RAW264.7 with poly I:C in the presence of pJAK2(1001–1013) resulted in significant enhancement of NO production. Classically, poly I:C induces type I IFNs and these in turn activate myeloid cells to produce NO, reflecting the activation of these cells (Matsumoto and Seya, 2008). In addition,

pJAK2(1001–1013) enhanced NO production in response to LPS, an activator of TLR4, suggesting that pJAK2(1001–1013) may have antibacterial properties.

A well recognized but not fully understood aspect of IFN function in cells is that most cells constitutively produce low levels of intracellular IFNβ that is thought to play a role in induction of an antiviral state in cells treated with type I and type II IFNs (Taniguchi and Takaoka, 2001). pJAK2(1001–1013) increased the level of intracellular IFNβ in cells where it induced the antiviral effect. Intracellular signaling by a non-secretable form of type I IFN has been reported by several independent studies (Ahmed et al., 2001; Shin-Ya et al., 2005). Both IFNα and IFNβ were expressed in a non-secretable form, and were able to induce an antiviral state without being secreted. Related to these findings, SOCS-1 deficiency has been shown to amplify type I IFN antiviral actions (Fenner et al., 2006). Additionally, type I IFNs activate STAT1, an effect that is increased by pJAK2(1001-1013) *in vitro*. Mice deficient in STAT1 are phenotypically normal when kept in a specific pathogen free environment, but they are extremely susceptible to viral and bacterial infection (Meraz et al., 1996). In keeping with the TLR findings of this study, it would be interesting to determine the effects of pJAK2(1001–1013) on the ability of $STAT1^{-/-}$ mice to clear viral or bacterial infection.

The SOCS-1 antagonist exerted a direct effect and synergized with IFNγ and IFNγ(95–132) to protect cells and mice from EMCV infection. These observations, along with the inhibition of HSV-1 replication in keratinocytes and protection against vaccinia virus, demonstrate the broad antiviral activity of the SOCS-1 antagonist. The connection of the SOCS-1 antagonist with IFNγ appears to reduce the regulatory restraints imposed by SOCS-1 under normal physiological conditions as shown by SOCS-1 reduction in

cells treated with pJAK2(1001–1013). The mechanism of the reduction is not currently known, but may be related to proteasomal degradation via the SOCS box of SOCS-1.

Targeting of SOCS-1 expression in cardiac myocytes by expression of a dominant negative SOCS-1 increased myocyte resistance to acute cardiac injury as well as reduced mortality in coxsackievirus-infected mice (Yasukawa, 2003). This transfection study with a generic approach to inhibition of SOCS-1 function is analogous to the SOCS-1 antagonist results presented here. These results provide an approach to targeting SOCS-1 with a flexible drug therapeutic potential. Induction of SOCS-1 in other viral infections such as influenza (Pothlichet et al., 2008) further suggests a role for the SOCS-1 antagonist in modulation of these infections.

There has been great interest in developing small molecule agonists or inhibitors of various cytokines and cytokine regulators. This study presents both, in the form of SOCS-1 mimetics and antagonists. The SOCS-1 mimetics are able to emulate the biological activity of SOCS-1, and this property can be used to treat a variety of immunological diseases that have a disregulation in the JAK/STAT signaling pathway, as well as certain viral infections. We have recently revealed a novel endogenous antipoxviral activity of SOCS-1 (Ahmed et al., 2009). The SOCS-1 mimetics prevent vaccinia virus from hijacking the host's replication machinery by blocking the activity of the ErbB1 tyrosine kinase, a kinase that is necessary for viral replication and release (Buller et al., 1988). Additionally, the SOCS-1 mimetics inhibited virus-induced activation of JAK2, similar to the inhibition of myxoma virus infection in rabbits by tyrophostin AG490, a JAK-specific inhibitor (Masters et al., 2001).

The SOCS-1 antagonist pJAK2(1001–1013) is able to function as a broad spectrum antiviral, as it is effective against vaccinia virus, HSV-1, and EMCV. It would be interesting to test its effects on parasitic infections, as SOCS-$1^{-/-}$ mice are resistant to not only viral infections, but certain parasitic infections as well (Zimmermann et al., 2006). *Toxoplasma gondii* parasites are able to block IFNγ signaling pathways via induction of SOCS-1, and this enables the parasite to evade the immune response and live and replicate happily in macrophages (Zimmermann et al., 2006). The ability of the parasite to interfere with IFNγ signaling was abolished when macrophages from mice deficient in SOCS-1 were infected with *T. gondii*, suggesting that the parasite induces endogenous SOCS-1.

Future studies will focus on further elucidating the complete mechanisms of action of the SOCS-1 mimetic and antagonist peptides, including looking closer into their physiological roles. The SOCS-1 mimetics will be used to treat SOCS-1 knockout mice and their effects on the inflammatory pathology, as well as their direct effects on the various cell types including B cells and T regulatory cells, will be determined. The SOCS-1 antagonist peptide will be used as a potential antiviral therapy for influenza A virus. Its effects on virus replication and infection *in vitro* will be determined, followed by *in vivo* studies in a mouse model of influenza A infection.

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BIOGRAPHICAL SKETCH

Rea Dabelic was born in Dubrovnik, Croatia, to Angie and Claudio Dabelic. She spent the first eight years of her life there, until the civil war broke out in early summer of 1991. Rea's family was one of the lucky families to escape the war on the last airplane to leave the country. Their flight took them to the Netherlands, where a loving family took them into their home. There, Rea spent the next year learning Dutch and adjusting to a new culture.

In August of 1992, Rea and her family emigrated to Coral Springs, Florida and they made that their permanent home. Rea spent the next nine years of her life there and graduated from J.P. Taravella High School in 2001. Upon graduating, Rea enrolled in the University of Florida, where she earned her Bachelor of Science in integrative biology in 2005.

Rea earned her Doctor of Philosophy degree in microbiology and cell science from the University of Florida in May of 2010. She plans to pursue a career in immunological research.