SUPPRESSORS OF CYTOKINE SIGNALING-1 (SOCS-1) MIMETIC AND ANTAGONIST PEPTIDES: POTENTIAL AS THERAPEUTIC AGENTS FOR TREATMENT OF IMMUNOLOGICAL DISEASES

By

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To the memory of my grandmother Mrs. Priscilla Wangechi Matu and my grandfather Mr. Richard Mwaniki wa Nyangi

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

SUPPRESSORS OF CYTOKINE SIGNALING –1 (SOCS-1) MIMETIC AND ANTAGONIST PEPTIDES: POTENTIAL AS THERAPEUTIC AGENTS FOR TREATMENT OF IMMUNOLOGICAL DISEASES

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Suppressor of cytokine signaling (SOCS)-1 protein modulates signaling by interferon gamma (IFNγ) by binding to the autophosphorylation site of Janus kinase 2 (JAK2) and by targeting bound JAK2 to the proteosome for degradation. Studies on a tyrosine kinase inhibitor peptide, Tkip, which is a SOCS-1 mimetic, are described. Tkip was synthesized from the physiological form of amino acids, L-amino acids. We synthesized two additional SOCS-1 mimetic peptides, DTkip and DRTkip, from D-amino acids, which are potentially more resistant to degradation and therefore would likely be better therapeutic agents. DTkip and DRTkip bound to the unphosphorylated JAK2 autophosphorylation site peptide, JAK2(1001-1013) and the tyrosine 1007 phosphorylated peptide, pJAK2(1001-1013). Further, DTkip and DRTkip inhibited JAK2 autophosphorylation and JAK2 phosphorylation of IFNγ receptor-1 (IFNGR-1).

Tkip was also compared with the kinase inhibitory region (KIR) of SOCS-1 for JAK2 recognition, inhibition of kinase activity, and regulation of IFNγ-induced biological activity. Tkip and a peptide corresponding to the KIR region of SOCS-1, ^{53}DTHFRTFRSHSDYRRI (SOCS1-KIR), bound similarly to JAK2(1001-1013) and to pJAK2(1001-1013). Dose-response competitions suggested that Tkip and SOCS1-KIR similarly recognized the autophosphorylation

site of JAK2. While Tkip inhibited JAK2 autophosphorylation as well as IFNγ-induced STAT1α phosphorylation, SOCS1-KIR, like SOCS-1, did not inhibit JAK2 autophosphorylation but inhibited STAT1α activation. Both Tkip and SOCS1-KIR inhibited IFNγ activation of murine macrophages and antigen-specific splenocyte proliferation.

The fact that SOCS1-KIR bound to pJAK2(1001-1013) suggested that the JAK2 peptide could function as an antagonist of SOCS-1. Thus, pJAK2(1001-1013) enhanced suboptimal IFNγ activity, blocked SOCS-1 induced inhibition of STAT3 phosphorylation in IL-6-treated cells, enhanced IFNγ activation site (GAS) promoter activity, and enhanced antigen-specific proliferation. Further, SOCS-1 competed with SOCS1-KIR for pJAK2(1001-1013). Thus, the KIR region of SOCS-1 binds directly to the autophosphorylation site of JAK2 and a peptide corresponding to this site can function as an antagonist of SOCS-1.

In summary, Tkip and SOCS1-KIR recognized the autophosphorylation site of JAK2 similarly and pJAK2(1001-1013) peptide functioned as a SOCS-1 antagonist. Thus, we have developed peptides that function as SOCS-1 agonists and antagonists, which have potential for suppressing or enhancing the immune response.

CHAPTER 1
INTRODUCTION

Janus tyrosine kinases (JAKs) are an enzyme family that mediates the biological effects of cytokines, hormones, and growth factors by tyrosine phosphorylation of signal transducers and activators of transcription, STATs (Reviewed in Yamoaka et al. 2004; Parganas et al. 1998). The interferons (IFNs), including types I and type II, hormones such as growth hormone and angiotensin, and growth factors such as thrombopoietin, are all among 50 related factors dependent on JAK tyrosine phosphorylation of appropriate STAT transcription factors for their physiological functions (Subramaniam et al. 2001; Johnson et al. 2004).

The immediate early signal transduction events associated with IFNγ and its receptor subunits involve the obligatory action of two tyrosine kinases, JAK1 and JAK2 (Reviewed in Kotenko and Peska 2000). The IFNγ receptor (IFNGR) system is a heterodimeric complex consisting of an α-subunit (IFNGR-1) and a β subunit (IFNGR-2), both of which are essential for the biological activity of IFNγ (Kotenko and Peska 2000). JAK1 is associated with the IFNGR-1 chain, whereas JAK2 is associated with the IFNGR-2 chain. The interaction of IFNγ, primarily with the IFNGR-1 subunit, initiates a sequence of events that results in increased binding of JAK2 to IFNGR-1. This has important consequences for subsequent critical phosphorylation events. JAK2, in the process of binding to IFNGR-1, undergoes autophosphorylation, and at the same time IFNGR-1 is phosphorylated. These events occur in concert with JAK1 function, resulting in recruitment and tyrosine phosphorylation of the IFNγ transcription factor, STAT1 (Reviewed in Kotenko and Peska 2000; Bromberg and Darnell 2000).

A family of proteins called suppressors of cytokine signaling (SOCS) negatively regulates JAK/STAT signaling (Starr et al. 1997; Endo et al. 1997; Naka et al. 1997). SOCS proteins are also negative regulators of signaling by other cytokines, growth factors, and hormones

(Reviewed in Alexander et al. 2002; Larsen and Ropke 2002; Alexander and Hilton 2004). There are currently eight identified members of the SOCS family, SOCS-1 to SOCS-7 and cytokine-induced SH2 domain containing protein (CIS). SOCS-1 is of particular interest, because it is a negative regulator of the JAK kinases, as well as several cytokines, and hormone receptor systems including epidermal growth factor receptor (EGFR) (Reviewed in Calo et al. 2003).

Our laboratory recently designed and synthesized a tyrosine kinase inhibitor peptide, Tkip (WLVFFVIFYFFR) (Flowers et al. 2004). The characteristics of the Tkip 12-mer are summarized in Chapter 2. In this study we show that Tkip inhibits IFNγ-induced macrophage activation and define Tkip cellular targets in antigen-induced immune responses. We also describe a family of Tkip-related peptides, DTkip (WLVFFVIFYFFR) and DRTkip (RFFYFIVFFVLW). DTkip and DRTkip, which like Tkip, bind to the JAK2 autophosphorylation site peptide, inhibit JAK2 autophosphorylation, and JAK2 phosphorylation of IFNγ receptor subunit, IFNGR-1.

It has been suggested that the binding of SOCS-1 to JAK2 requires the SOCS-1 SH2-domain and that the kinase inhibitory region (KIR), while not required for the binding, is essential for the inhibitory action of SOCS-1 (Yasukawa et al. 1999). We show here that a peptide corresponding to SOCS-1 KIR region, SOCS1-KIR, specifically binds to a peptide representing the JAK2 autophosphorylation site and inhibits STAT1α activation. Further, we show that SOCS1-KIR, as well as Tkip, inhibit IFNγ-induced macrophage activation. We also present data on a novel SOCS-1 antagonist peptide, pJAK2(1001-1013), which corresponds to the JAK2 autophosphorylation site. pJAK2(1001-1013) enhances suboptimal IFNγ-induced antiviral activity, enhances IFNγ-activated sequences (GAS) promoter activity, and inhibits

SOCS-1 suppression of STAT3 activation of LNCaP prostate cancer cells, thus functioning as a SOCS-1 antagonist peptide.

Rationale

SOCS proteins play an important role in the regulation of JAK/STAT signaling. Disruption of the normal SOCS function may contribute to disease onset, progression or death. It has been shown that deletion of SOCS-$1^{-/-}$ or SOCS-$3^{-/-}$ genes in mice results in death of the mice either as neonates (SOCS-$1^{-/-}$) or embryos (SOCS-$3^{-/-}$) (Naka et al. 1998; Starr et al. 1998). SOCS proteins regulate immune response by inhibiting JAK kinases. Uregulated JAK kinases signaling may result in inflammation and cancers.

Several cancers are characterized by constitutive activation of the JAK/STAT signaling pathway. SOCS proteins may play a role in inhibiting malignant transformation of cells by regulating JAK/STAT signaling, thereby preventing cancer onset and progression. This has been shown in a number of cancers including leukemia (T cell acute lymphoblastic leukemia (ALL), Pre-B ALL, and atypical chronic myelogenous leukemia (CML)), and hepatocellular carcinoma (Alexander and Hilton 2004). These examples raise the interesting possibility of the role that SOCS proteins and/or SOCS mimetics may play in the management of these cancers.

Dysregulation of JAK/STAT signaling also plays important roles in the pathogenesis of some inflammatory diseases including rheumatoid arthritis (Suzuki et al. 2001), inflammatory diseases of the gastrointestinal tract (SOCS2), (Suzuki et al. 2001; Lovato et al. 2003; Shouda et al. 2001) as well as inflammations of the central nervous system for example in experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis in humans (Maier et al. 2002). Hence, dysregulation of JAK/STAT signaling plays an important role in the pathology of some inflammatory diseases and therefore raises the possibility that SOCS proteins and/or SOCS

mimetics that negatively regulate JAK/STAT signaling may be possible therapeutics for the control and treatment of the inflammatory disease.

There are several tyrosine inhibitors that are currently undergoing clinical trials for treatment of various cancers, especially cancers resistant to chemotherapy and radiation. Herceptin (Trastuzumab) is a humanized monoclonal antibody specific for a member of the epidermal growth factor receptor (EGFR) family called HER2, which binds to the extracellular binding domain of HER2/neu on tumor cells inducing receptor internalization and inhibiting cell cycle progression (Reviewed in Shawver et al. 2002). The discovery of Herceptin resulted in the treatment of aggressive forms of breast cancer, in which cancer cells overexpressed HER2. These cancers are usually less responsive to chemotherapy (Shawver et al. 2002). A second drug, Gleevac^{TM}, is a small molecule inhibitor of the oncogenes BCR-Abl, Abl, PDGFR, and c-kit. Gleevac^{TM} blocks ATP binding to the kinase, thereby preventing phosphorylation events that are required for signal transduction. Gleevac^{TM} has been shown to increase the effectiveness of interferon therapy on chronic myelogenous leukemia patients (Shawver et al. 2002). Other small molecule tyrosine kinases targeted therapies include Erbitux (EGFR) for treatment of colorectal cancer, Tarceva (EGFR) for treatment of pancreatic cancer, and Iressa (EGFR) for treatment of non-small-cell lung cancer (Shawver et al. 2002; Vincentini et al. 2003). This provides additional evidence that tyrosine kinases targeted approaches may have potential as anti-cancer therapy.

Hence, it is possible that the SOCS-1 mimetic peptides developed in our laboratory, which are tyrosine kinase inhibitors of STAT transcription factors such as the STAT3 oncogene, may have potential as anti-cancer and anti-inflammatory disease agents. The mimetics would likely augment the effect of endogenous SOCS-1. We will discuss these mimetic peptides and present data on their effect on the JAK/STAT signaling pathway, IFNγ-signaling, and cell proliferation.

SOCS-1 regulates signaling by a variety of cytokines including IFNγ, IFNα, and several interleukins. Put differently, SOCS-1 reduces the immune response mechanisms initiated by these cytokines. In an immune competent individual, this is desirable because it prevents excessive signaling, which would likely result in inflammation. However, in an immunocompromised individual, there may be a need to enhance cytokine-induced immune response to infection. One way of enhancing immune response to pathogen infection may be by inhibition of SOCS-1 activity. It has recently been shown that silencing of SOCS-1 in dendritic cells promoted cell activation, which led to enhancement of effective antigen-specific anti-tumor immunity (Shen et al. 2004). This implies that reagents that negatively regulate SOCS-1 may have potential in control and/or treatment of diseases arising from inadequate immune response. Hence, we designed a SOCS-1 inhibitor peptide, pJAK2(1001-1013), that is derived from the JAK2 autophosphorylation site. I will present data showing that pJAK2(1001-1013) is a SOCS-1 antagonist.

Specific Objectives

In this dissertation Tkip and other SOCS-1 mimetic peptides, which I hypothesized, would function similar to Tkip and therefore similar to SOCS-1 are described. The first objective of this study therefore was to determine whether these peptides; DTkip and DRTkip have Tkip-related characteristics. Specifically, whether the peptides bind to JAK2 autophosphorylation site, inhibit JAK2 autophosphorylation, and inhibit activated JAK2 activity.

It has previously been stated that the SOCS-1 domains that bind directly to JAK2 autophosphorylation site are the SH2 domain and the extended SH2 region (ESS), and that the kinase inhibitory region (KIR) is not essential in the initial binding (Yasukawa et al. 1999). The second objective of this study was therefore to determine whether a peptide corresponding to

SOCS-1 KIR, SOCS1-KIR, binds directly to JAK2 autophosphorylation site, inhibits JAK2 autophosphorylation, and inhibits activated JAK2 activity.

Since Tkip has been shown to be a SOCS-1 mimetic with possible therapeutic potential, the third objective of this study was to define Tkip cellular targets. Specifically, to determine whether Tkip targeted B cells, $CD4^+$ T cells, $CD8^+$ T cells, and antigen presenting cells. This would provide insights on the direct effect of Tkip on specific cells of the immune system, which is important if Tkip proves be a potential therapeutic agent.

The fourth objective of this study was to define a novel way of addressing inadequate cytokine-induced immune response mechanism. Here, a novel SOCS-1 antagonist peptide, pJAK2(1001-1013), is described and preliminary data showing that this peptide reverses SOCS-1 inhibition is presented.

CHAPTER 2
LITERATURE REVIEW

Janus Kinases

Janus tyrosine kinases (JAKs) are a small but indispensable enzyme family of nonreceptor tyrosine kinases that mediates the biological effects of cytokines, hormones, and growth factors by tyrosine phosphorylation of signal transducers and activators of transcription, STAT, (Reviewed in Ihle 1995; Parganas et al. 1998). The JAK family consists of four members (JAK1, JAK2, JAK3, and TYK2) that are differentially activated in response to various cytokines (Ihle 1995). JAK proteins are approximately 120 to 130 kDa cytosolic proteins expressed in many types of tissue with the exception of JAK3 whose expression is restricted to cells of the hematopoietic system (Ihle 1995, Reviewed in Thompson 2005).

Members of the JAK protein family contain highly conserved structural domains designated JAK homology domains (JH). There are currently seven known JH domains (JH1-JH7) of which JH1 is the functional catalytic kinase. For JAK2, this domain possesses a critical activation loop that becomes phosphorylated in order to activate the kinase. The phosphorylation of tyrosine residue (Y1007) in the activation loop of JAK2 is essential for activation and downstream signaling events (Feng et al. 1997). It has recently been suggested that the phosphorylation of other tyrosines may also be necessary for JAK2 activation (Kurzer et al. 2004). Phosphorylation of Y1007 results in a conformational change in the activation loop, which allows substrate access to specific binding sites in the catalytic groove (Yasukawa et al. 1999).

JH2 is a non-functional catalytic kinase domain but it bears sequence homology to typical tyrosine kinase domains, hence it is referred to as a pseudokinase domain. The functional role of the JH2 domain is unclear, however studies have suggested that it may have a kinase inhibitory

function. Both the JH1 and JH2 domains are located near the carboxyl terminus and comprise the major portion of the JAK molecule (Reviewed in Leonard 2001; Kisseleva et al. 2002). The JH3, JH4, and JH5 domains are poorly understood and require additional work to elucidate their functions. JH6 and JH7 amino terminal domains have been implicated in the association between the JAK molecule and the specific cytokine receptor (Leonard 2001; Kisseleva et al. 2002). Figure 2-1 shows a diagrammatic representation of the domain structure of the JAK kinases.

Comparisons of the JAK2 autophosphorylation site amino acid sequences (LPQDKEYYKVKEP) revealed100% sequence homology among different mammalian species including of the human JAK2 (*Homo sapiens*, genebank accession number NM_004972) mouse (*Mus musculus*, AAH54807), rat (*Rattus norvegicus*, NP_113702), and pig (*Sus scrofa*, BAA21662) as determined using the basic local alignment search tool (BLAST search, http://www.ncbi.nlm.nih.gov/blast/). In addition, the amino acid sequence of the human JAK1 (IETDKEYYTVKDD, accession number NP_002218) was 100% homologous to the mouse JAK1 (NP_666257), as was human TYK2 (VPEGHEYYRVRED，accession number NP_003322） and mouse TYK2 (NP_061263). Human JAK3 (LPLDKDYYVVREP, NP_000206) and mouse JAK3 (LPLGKDYYVVREP, NP_034719), are nearly identical with a one amino acid substitution, underlined. It is worth noting that in all four JAK kinases, the sequences are similar, but not identical. The conservation in this region indicates the importance of the autophosphorylation site in JAK function.

Signal Transducers and Activators of Transcription (STAT) Proteins

STAT proteins are a family of cytoplasmic transcription factors that participate in a variety of cellular events, including differentiation, proliferation, cell survival, apoptosis, and angiogenesis involving cytokines, growth factors, oncogenes, and hormones. Some of the

cytokines, growth factors, oncogenes, and hormones that utilize STAT proteins are shown in Table 2-1.

There are currently seven known STAT family members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Darnell 1997). We are particularly interested in STAT1 and STAT3, both of which are negatively regulated by SOCS-1. It is worth noting that other SOCS proteins may also negatively regulate STAT1 and STAT3.

The STAT proteins are comprised of six domains. These are oligomerization, coiled-coil, DNA binding, linker, SH2, and transcription activation domains. The binding of STAT to the receptors occurs through interaction of the SH2 to the receptor-docking site. The critical tyrosine residues required for SH-phosphotyrosine interaction are STAT1-Y701; STAT2-Y690; STAT3-Y705; STAT4-Y693; STAT5-Y694, and STAT6-Y641. These phosphotyrosines are located near the SH2 domain (Caló et al. 2003).

The linker domain, which is alpha helical is the bridge between the DNA binding and the SH2 domains. The transcription activation domain, located on the carboxyl terminus is involved in communication with transcription complexes. The domain has a conserved serine residue (except in STAT2 and STAT6) that when phosphorylated regulates STAT transcription activity (Reviewed in Imada and Leonard 2000). The amino terminus region of STAT proteins is highly conserved and provides protein to protein interaction, such as dimer interaction of STAT molecules, which contributes to the stability of STAT-DNA binding, thereby increasing transcription activity (Imada and Leonard 2000). The coiled-coil domain may be involved in regulatory function and may be responsible for nuclear export of STAT.

Although some constitutively activated STATs have been observed in some human cancer cell lines and primary tumors, STAT proteins are generally activated by tyrosine phosphorylation

(Caló et al. 2003). The STAT proteins become activated by a variety of receptors, such as cytokine, growth factor, and hormone receptors, which may activate STATs directly or indirectly through JAK kinases. In JAK/STAT signaling, the binding of a cytokine to its receptor results in the associated JAK kinase becoming phosphorylated, and hence activated. The activated JAK kinase phophorylates the receptor's cytoplasmic domain, at specific tyrosine residues, and opens docking sites for STAT proteins. The STAT proteins docked on the receptor, are phosphorylated, and thus become activated. Some models of JAK/STAT signaling indicate that the activated STATs dissociate from the receptor, dimerize, and translocate to the nucleus where they activate the transcription of specific genes (Bromberg and Darnell 2000). However, it has been shown that in the case of IFNγ signaling, IFNγ, IFNGR-1, and the bound phospho-STAT1 dimer are translocated to the nucleus as a complex (Subramaniam et al. 2001, Ahmed et al. 2003, Ahmed and Johnson 2006), which implies that IFNGR-1 likely plays additional roles in signal transduction.

Interferon Signaling Through the JAK/STAT Signaling Pathway

There are two classes of interferon, type I and type II, both of which utilize the JAK/STAT signaling pathway. Although type I and type II IFNs signaling pathways are similar, distinct receptors, JAK kinases, and STAT proteins are utilized. IFNγ, the type II IFN, signaling utilizes IFNGR, which is a heterodimeric complex comprised of two subunits, α (IFNGR-1) and β (IFNGR-2), both of which are essential for biological activity of IFNγ (Kotenko and Pestka 2000).

IFNGR-1 is associated with JAK1, while the IFNGR-2 is associated with JAK2. Interaction of IFNγ, primarily with IFNGR-1, causes the receptor subunits to dimerize and brings the associated JAK1 and JAK2 into close proximity. The JAK kinases undergo

autophosphorylation at specific tyrosine residues (1007 for JAK2) and become activated (Kotenko and Pestka 2000). The activated JAKs phosphorylate and activate the IFNGR subunits, which results in a cascade of events including the phosphorylation of STAT1α. Two phosphorylated STAT1 monomers dimerize and are phosphorylated at specific serine residues to form an activated STAT1 transcription factor. According to some models of IFNγ signaling, the STAT1 dimers dissociate from the IFNGR-1, translocate to the nucleus, bind to IFNγ activation sites (GAS), and induce expression of target genes (Bromberg and Darnell 2000). It has however been shown that IFNGR-1 accumulates in the nucleus and colocalizes with STAT1α in a time and dose dependent manner, implying that IFNGR-1 is likely also translocated into the nucleus (Subramanian et al. 2001, Ahmed et al. 2003, Ahmed and Johnson 2006). Thus, IFNGR-1 may play an active role in signal transduction events subsequent to binding of the receptor complex. Studies have indicated that IFNGR-2 likely is not translocated to the nucleus (Ahmed and Johnson 2006).

Type I interferons, IFNα for example, utilize the interferon alpha-receptor (IFNAR) for signal transduction. The IFNAR is comprised of two subunits, IFNAR-1 (associated with TYK2) and IFNAR-2 (associated with JAK1) (Kotenko and Pestka 2000). The signal transduction pathway is initiated when IFNα binds to the receptor, which results in IFNAR-1 and IFNAR-2 forming a heterodimer with subsequent autophosphorylation and activation of both JAK1 and TYK2. The activated JAK kinases phosphorylate IFNAR-2, providing docking sites for STAT2, which binds to the receptor and becomes phosphorylated at tyrosine 690. The phosphorylation favors the binding of STAT1 to the phosphorylated STAT2 (Durbin et al. 1996). STAT1 is phosphorylated (tyrosine 701) by the JAK kinases and the STAT2/STAT1 heterodimer is released from the receptor and translocates to the nucleus. The STAT2/STAT1 heterodimer

associates with p48 nuclear factor to form the IFN-stimulated gene factor (ISGF3) complex (Horvath et al. 1996), which stimulates activation of target genes within the IFN-stimulated response elements (IRSE) (Li et al. 1998).

Regulation of the JAK/STAT Signaling Pathway

The JAK/STAT signaling pathway is carefully regulated. Unregulated JAK/STAT signaling may result in excessive cytokine-induced immune response, which would likely result in inflammation and be harmful to cells, and ultimately to the organism. Three classes of regulators of the JAK/STAT pathway are currently known. These are the protein inhibitors of activated STATs (PIAS), tyrosine phosphatases that include the Src-homology 2 (SH2)-containing protein tyrosine phosphatases (SHPs), and the suppressors of cytokine signaling protein family (SOCS) (Reviewed in Kile et al. 2001; Kisseleva 2002; Larken and Röpke, 2002, Alexander and Hilton 2004). PIAS proteins regulate transcription through several mechanisms, including blocking the DNA-binding activity of transcription factors, recruiting transcriptional co-repressors and promoting protein sumoylation (Shuai 2006). SHPs regulate JAK/STAT pathway by dephosphorylating activated phosphotyrosine (Reviewed in Rico-Bautista et al. 2006). SOCS can block cytokine signaling by acting as (i) kinase inhibitors of JAK proteins (SOCS1 and SOCS3), (ii) binding competitors against STATs (SOCS3 and CIS) and (iii) by acting as ubiquitin ligases, thereby promoting the degradation of their partners (SOCS1, SOCS3, and CIS) (Rico-Bautista et al. 2006). I will discuss the SOCS proteins, in detail, as an example of negative regulators of JAK/STAT signaling, because SOCS proteins are essential for the regulation of JAK/STAT signaling and other signaling pathways.

Suppressors of Cytokine Signaling (SOCS)

SOCS proteins are a family of cytoplasmic proteins that negatively regulate signal transduction of cytokines, hormones, and growth factors that utilize the JAK/STAT signaling

pathway (Naka et al. 1997, Starr et al. 1997, Endo et al. 1997). Loss or insufficient expression of SOCS proteins may result in diseases including several immune disorders, inflammatory diseases, and cancers (Reviewed in Alexander and Hilton 2004; Tan and Rabkin 2005). Mouse studies have shown that deletion of the SOCS-1 gene results in neonatal death (Naka et al. 1998; Starr et al. 1998), while deletion of the SOCS-3 gene results in embryonic death (Croker et al. 2003). In the case of human disease, it has been shown that a number of hematological malignancies are characterized by constitutive activation of JAK/STAT signaling pathway. These malignancies include T cell acute leukemia and atypical chronic myelogenous leukemia (Alexander and Hilton 2004). Further, SOCS-1 is likely a tumor suppressor since aberrant DNA methylation of SOCS-1 gene, resulting in transcriptional silencing, has been observed in human hapatocellular carcinomas and hepatoblastomas. The restoration of SOCS-1 expression in cells in which SOCS-1 gene had been silenced led to reduction in the transformed phenotype (Alexander and Hilton 2004), providing direct evidence that lack of SOCS-1 expression may have played a role in the development of the malignancies. In addition, constitutive activation of STAT3 and deregulation of SOCS3 expression have been observed in a variety of inflammatory diseases (Alexander and Hilton 2004). Thus, SOCS proteins play a fundamental role in maintaining health.

There are eight members of the SOCS family. These are the cytokine-induced SH2 domain containing protein (CIS), SOCS-1, SOCS-2, SOCS-3, SOCS-4, SOCS-5, SOCS-6, and SOCS-7 (Reviewed in Alexander 2002; Larsen and Röpke, 2002; Alexander and Hilton, 2004). The SOCS proteins are also known as JAK-binding protein (JAB), STAT-induced STAT inhibitor (SSI), and cytokine-inducible SH2 containing (CIS) proteins (Larsen and Röpke, 2002). All SOCS proteins have three shared domains, which are an N-terminal domain of varying length

and sequences, a central SH2 domain, which is essential for binding to the JH1 region of JAK kinases (Yasukawa et al. 1999), and a C-terminal SOCS box. The SOCS box couples substrate-specific interactions of the SH2 domain to the ubiqutination machinery, resulting in proteosomal degradation of associated JAK (Alexander 2002). SOCS-1 and SOCS-3 also have the kinase inhibitory region, KIR, which is hypothesized to be involved in catalytic activity of SOCS-1, but not in the actual binding to JAK2 autophosphorylation site (Yasukawa et al. 1999, Giordanetto and Kroemer 2003). In this manuscript, data will be provided indicating that the KIR region is likely involved in direct binding to JAK2 autophosphorylation site. Figure 2-2 contains a schematic representation of the domain structure of SOCS-1.

The physiological roles of four SOCS family members (CIS, SOCS-1, SOCS-2, and SOCS-3) are well defined. I will briefly discuss the physiological role of SOCS-1 as an example of the important role that SOCS proteins play in maintaining homeostasis.

Physiological Role of SOCS-1 Protein

SOCS-1 plays a vital role in negative regulation of IFNγ signaling as shown by both *in vivo* studies and *in vitro* assays. SOCS-1 double knockout mice (SOCS-$1^{-/-}$) die as neonates, displaying low body weight and liver damage including necrosis. Diseased livers are characterized by the presence of aggregates of granulocytes, eosinophils, and macrophages. The SOCS-$1^{-/-}$ mice also exhibit monocytic invasion of the pancreas, the lung, and the heart (Starr et al. 1998; Naka et al. 1998) and have a marked reduction in blood and spleen lymphocytes, as well as severe deficiencies in both mature B- and T lymphocytes. In addition, mice thymuses are reduced in size and show increased numbers of apoptotic cells both in the spleen and the thymus when compared to normal mice. These symptoms indicate severe deficiencies in the immune system (Naka et al. 1998; Starr et al. 1998) and show that SOCS-1 is indispensable for normal neonatal development.

The pathologies observed in SOCS-$1^{-/-}$ gene knock out mice were similar to those observed in wild-type mice administered excess IFNγ, which led to the hypothesis that the disease observed in the SOCS-$1^{-/-}$ gene knock out mice was likely due to excessive response to IFNγ. Direct evidence that IFNγ was required for the development of the lethal disease observed in SOCS-$1^{-/-}$ gene knock out mice was obtained when the mice were treated from birth with IFNγ neutralizing antibodies. After three weeks of IFNγ treatment the anti-IFNγ treated mice remained healthy, while all the untreated mice had succumbed to disease (Alexander et al. 1999). In addition, the SOCS-$1^{-/-}$/IFN$\gamma^{-/-}$ double knockout mice did not exhibit the lethal phenotype observed in SOCS-$1^{-/-}$ knockout mice (Alexander et al. 1999).Thus, SOCS-1 is a key physiological regulator of IFNγ signaling. It is worth noting that SOCS-$1^{-/-}$/IFN$\gamma^{-/-}$ double knockout mice eventually died 6 months after birth with inflammation and polycystic kidneys (Metcalf et al. 2002), which suggested that SOCS-1 regulation was not specific for IFNγ.

The prediction that SOCS-1 regulates IFNγ signaling was confirmed using biochemical studies. Upon injection of IFNγ into mice, STAT1 phosphorylation was evident in the livers of the SOCS-$1^{+/+}$ mice within 15 min but declined after 2 h. However, in the SOCS-$1^{-/-}$ gene knock out mice, phosphorylated STAT1 remained and was detectable 8 h after IFNγ administration (Brysha et al. 2001), indicating continued IFNγ signaling. These observations, taken together, indicated that SOCS-1 is a physiological negative regulator of IFNγ signaling (Alexander and Hilton 2004) and that unregulated IFNγ activity contributed to the pathology observed in the SOCS-$1^{-/-}$ gene knock out mice. Thus, SOCS-1 plays a fundamental role in regulating IFNγ signal transduction. It has also been shown that SOCS-1 plays a fundamental role in the regulation of IFNα and IFNβ (Fenner et al. 2006) and other cytokines signaling through the JAK/STAT pathway.

SOCS-1 Mimetic Peptides

Our laboratory has designed a family of SOCS-1 mimetic peptides, which are being tested for SOCS-1-like activity. The first of these peptides was tyrosine kinase inhibitor peptide (Tkip, WLVFFVIFYFFR), which was designed using a complementary peptide approach for complementarity to the JAK2 autophosphorylation site (Flowers et al 2004). Tkip binds to the JAK2 autophosphorylation site and inhibits JAK2 autophosphorylation and JAK2 mediated phosphorylation of the IFNGR-1 (Flowers et al. 2004). Tkip also inhibits the autophosphorylation of the epidermal growth factor receptor (EGFR), consistent with the fact that EGFR is regulated by SOCS-1 and SOCS-3. In contrast, Tkip does not bind or inhibit tyrosine phosphorylation of the vascular endothelial growth factor receptor (VEGFR) or the substrate peptide of the protooncogene, c-Src (Flowers et al. 2004), both of which are not regulated by SOCS-1 suggesting specificity of Tkip-mediated inhibition. Although Tkip binds to unphosphorylated JAK2 autophosphorylation site peptide, JAK2(1001-1013), it binds significantly better to phosphorylated JAK2 autophosphorylation site peptide, pJAK2(1001-1013). It has been suggested that SOCS-1 recognizes and binds only to phosphorylated JAK2, therefore Tkip recognizes the JAK2 autophosphorylation site similar to SOCS-1, but not in precisely the same way. Consistent with inhibition of JAK2, Tkip also inhibits the ability of IFNγ to induce an antiviral state as well as upregulation of MHC class I molecules, and blocks the phosphorylation of both STAT1 and STAT3 (Flowers et al. 2004). Tkip also inhibits the proliferation of the prostate cancer cell lines DU145 and LNCaP, in a dose dependent manner (Flowers et al. 2005). In addition, Tkip has been shown to protect mice from EAE, an animal model for the human inflammatory disease, multiple sclerosis (Mujtaba et al. 2005) via blockage activation of inflammatory cytokines. Hence, Tkip appears to have both anti-inflammatory and anti-tumor properties.

Tkip is synthesized from L-amino acids, which are the physiological form of amino acids in nature and therefore are potentially readily degraded by proteases. Hence, if Tkip were to be used as a therapeutic agent, there is potential that it may be readily degraded. This would likely limit Tkip usefulness as a therapeutic agent. To address this limitation, we synthesized other Tkip-related mimetic peptides. These were DTkip (WLVFFVIFYFFR) and DRTkip (RFFYFIVFFVLW), which were synthesized from D-amino acids, which from a medicinal chemistry view, were likely to be more resistant to proteolytic digestion and therefore likely to be better therapeutics. DTkip has the same amino acid sequence as Tkip, while in DRTkip the sequence is reversed, in other words DRTkip is a retro-inversion of Tkip. DTkip and DRTkip were tested for SOCS-1-like activity such as the ability to bind to JAK2, inhibit JAK2 autophosphorylation, and inhibit JAK2-mediated phosphorylation of the substrates of the JAK/STAT pathway. In addition, the peptides were also tested for effects on cell proliferation, IFNγ-induced antiviral activity and upregulation of MHC class I molecules. In addition some Tkip cellular targets were identified. I will present data describing a family of SOCS-1 mimetic peptides and present a proof-of-concept for the therapeutic potential of Tkip.

Inhibition of SOCS-1 Activity and Immunological Relevance

As stated earlier, SOCS-1 is a negative regulator of immune factors including IFNs, interleukins –2, -3, -4, -6, -7, and –12, tumor necrosis factor (TNFα) as well as a variety of hormones such as growth hormone (Tan and Rabkin 2005). This raises the possibility that one way of enhancing cytokine-mediated immune response to pathogen infection may be by inhibition of SOCS-1 activity. It has recently been shown that silencing of SOCS-1 in dendritic cells promoted cell activation, which led to enhancement of effective antigen-specific anti-tumor immunity (Shen et al. 2004). This implies that reagents that regulate SOCS-1 may enhance immune responses and therefore have potential in control and/or treatment of diseases arising

from inadequate immune responses. A SOCS-1 inhibitor peptide, pJAK2(1001-1013), that is derived from the JAK2 autophosphorylation site was designed and synthesized. I will present data showing that pJAK2(1001-1013) is a SOCS-1 antagonist.

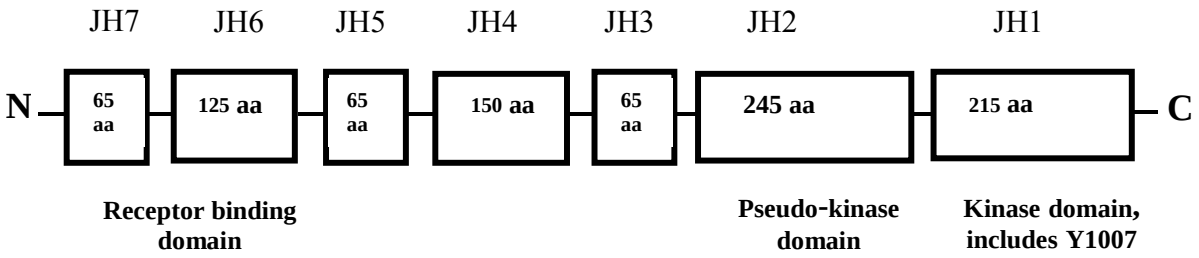


Figure 2-1. A schematic representation of the domain structure of the JAK kinase family. The JAK tyrosine kinase protein family contains four members: JAK1, JAK2, JAK3, and TYK2. Each JAK molecule contains seven distinct regions: JH1-JH7. The JH1 domain is the catalytic domain, which includes the activation loop, in which autophosphorylation site (Y1007) is located. The JH2 domain is the pseudo-kinase domain and is believed to play a role in the autoregulatory activities of JAK2. The JH6-JH7 domains mediate binding of JAK molecules to cytokine receptor proteins. Adapted with modifications from Imada and Leonard 2000.

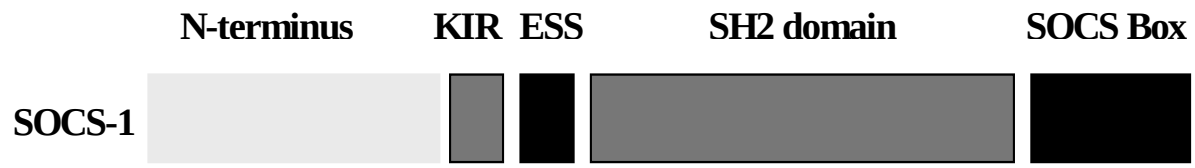


Figure 2-2. A schematic diagram showing the domain structure of SOCS-1 protein. All SOCS proteins have an N-terminal region of varying length and sequence, a central SH2 domain, and a C-terminal SOCS box. SOCS-1 and SOCS-3 have a kinase inhibitory domain (KIR), which lies between the N-terminal and the SH2-domain. In SOCS-1, the 12-amino acids N-terminal and contiguous to the SH2 domain form the extended SH2 (ESS) region, and the 12-amino acid residues N-terminal and contiguous with the ESS form the kinase inhibitory region (KIR). The SOCS1-KIR peptide is derived from the KIR, while the SOCS1-ESS peptide is derived from the ESS region.

Table 2-1. A table showing the JAK kinases and STAT proteins utilized by some cytokines, growth factors, hormones, and oncogenes.

Cytokines/hormones/oncogenes/ Growth factors	JAK	STAT
Cytokines		
IFN-γ	JAK1 and JAK 2	STAT 1
IFN-α/β	JAK1 and TYK-2	STAT 1, 2, 3, 4, 5A, 6
IL-2	JAK 1 and JAK 3	STAT 1, 3, 5A/5B
IL-3	JAK 2	STAT 1, 3, 5A/B, 6
IL-4	JAK 1 and JAK 3	STAT 6
IL-6	JAK 1	STAT 3, 5A/B
IL-10	JAK 1 and TYK-2	STAT 1, 3
IL-12	JAK 2 and TYK-2	STAT 1, 3, 4, 5
Growth factors/hormones		
EGF	JAK2	STAT 1, 2, 3, 5
Growth hormone	JAK2	STAT 1, 3, 5A/B
Insulin	JAK1	STAT3, STAT 5B
Oncogenes		
V-abl	JAK1	STAT 1, 3 and 5
V-src	JAK1 and JAK2	STAT 3

Adapted with modification from Subramaniam et al. 2001

CHAPTER 3
MATERIALS AND METHODS

Cell Culture

All the cell lines, except Sf9 insect cells, were obtained from the American Type Culture Collection (Manassas VA). The human prostate cancer cells, LNCaP, and the murine macrophage cells, Raw 264.7, were maintained in RPMI (JRH Biosciences, Lenexa, KS) supplemented with 10% FBS (Hyclone, Logan, CT), 100 U/mL penicillin, 100 U/mL streptomycin (complete media). Murine fibroblast cells, L929, were maintained in DMEM (JRH Biosciences), supplemented with FBS, penicillin, streptomycin, non-essential amino acids, sodium bicarbonate, and sodium pyruvate. The murine monocyte cell line, U937, was maintained in RPMI complete media supplemented with 10 mM HEPES (Sigma-Aldrich, St. Louis, MO), and 1 mM sodium pyruvate (Sigma-Aldrich). All the cell types were cultured at 37˚C and 5 % carbon dioxide humidified incubator. The Sf9 cells obtained from Invitrogen (Invitrogen Corporation, Carlsbald, CA) were maintained at 27^{o}C as adhesion cultures in complete TNM-FH media (Grace Insect Medium, Supplemented, containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin) or as suspension cultures in Sf-900 SFM media containing 5% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin.

Peptides

The peptides used in this study are listed in Table 3-1, and were synthesized by Mr. Mohammed Haider, in our laboratory on an Applied Biosystems 9050 automated peptide synthesizer (Foster City, CA) using conventional fluorenylmethyoxycarbonyl (Fmoc) chemistry as previously described (Szente et al. 1996). A lipophilic group (palmitoyl-lysine) was added to the N-terminus of peptides, to facilitate entry into cells, as a last step using a semi-automated protocol (Thiam et al. 1999). Peptides were characterized by mass spectrometry and where

possible, HPLC purified. Peptides were dissolved in water or in DMSO (Sigma-Aldrich, St. Louis, MO). The control peptides used in this project did not show significant biological activity in the systems tested.

Binding Assays

Binding assays were performed as previously described (Flowers et al. 2004) with minor modifications. Tkip, SOCS1-KIR, and control peptide, MuIFNγ(95-106) at 3 μg/well, were bound to 96-well plates, in binding buffer (0.1 M sodium carbonate and sodium bicarbonate, pH 9.6). The wells were washed three times in wash buffer (0.9% NaCl and 0.05% Tween-20 in PBS) and blocked in blocking buffer (2% gelatin and 0.05% Tween-20 in PBS) for 1 h at room temperature. Wells were then washed three times and incubated with various concentrations of biotinylated JAK2(1001-1013) or biotinylated pJAK2(1001-1013), in blocking buffer, for 1 h at room temperature. Following incubation, wells were washed five times and bound biotinylated peptides were detected using HRP-conjugated neutravidin (Molecular Probes) and color detected using *o*-phenylenediamine in stable peroxidase buffer (Pierce, 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 450-microplate reader (Bio-Rad Laboratories, Hercules, CA).

Peptide competition assays were carried out as described above except following peptide immobilization, washing, and blocking, biotinylated JAK2(1001-1013) or biotinylated pJAK2(1001-1013), which had been pre-incubated with various concentrations of soluble peptide competitors (Tkip, SOCS1-KIR or control peptide) was added. Detection of bound biotinylated peptide was conducted as described above. Data obtained from binding assays was plotted using Graph Pad Prism 4.0 software (Graph Pad Software, San Diego, CA).

In vitro Kinase Assays

Autophosphorylation activity of JAK2 was measured in a reaction mixture containing GST-JAK2 kinase fusion protein (Cell Signaling Technology, Danvers, MA), ATP (20 μM, Cell Signaling) and the appropriate peptide in kinase buffer (10 mM HEPES, pH 7.4, 50 mM sodium chloride, 0.1 mM sodium orthovanadate, 5 mM magnesium chloride, and 5 mM manganese chloride). It had previously been determined that soluble IFNGR-1 enhanced JAK2 activity (Flowers et al. 2004), therefore IFNGR-1 (2 μg/ reaction) was added and the reaction mix incubated at 25°C for 30 min with intermittent agitation. The assays were carried out according to a JAK2 kinase protocol obtained from Cell Signaling (Cell Signaling Technology, Danvers, MA), but with modifications, derived in part from Flowers et al. (2004). The reactions were terminated by addition of appropriate volume of 6 X SDS-PAGE loading buffer (0.5 M Tris-HCl (pH 6.8), 36% glycerol, 10% SDS, 9.3% DTT, 0.012% bromophenol blue), and heating at 95°C for 5 min. The proteins were separated on a 12% SDS-polyacrylamide gel (Bio-Rad Laboratories), transferred onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ), and probed with anti-pJAK2 antibodies (Santa Cruz Biotechnology, San Diego, CA). Membranes were then stripped and re-probed with anti-JAK2 antibody (Santa Cruz Biotechnology). Detection of proteins was accomplished using ECL protein detection reagents (Amersham Biosciences).

Autophosphorylation activity of epidermal growth factor receptor (EGFR, Upstate) was measured in a 50 μl reaction containing kinase buffer, 0.2 μg EGFR (Upstate Biotechnology), 0.1 μg EGF (Upstate Biotechnology), 20 μM ATP (Cell Signaling), and the appropriate peptide (50 μM) as described in Flowers et al. (2004). The reaction mix was incubated for 30 min at 25^0C, resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and

immunoblotted with anti-pEGFR antibody. The membranes were then stripped and reprobed with anti-EGFR antibody. Detection of proteins was accomplished using ECL protein detection reagents (Amersham Biosciences).

Immunoblot Analysis

U937 murine fibroblast cells were plated on 6-well plates at a cell density of 1 x 10^6 cells/ well and after an overnight incubation at 37°C and 5% CO_2, the cells were cultured in complete media containing varying concentrations of lipophilic Tkip, lipophilic SOCS1-KIR, or lipophilic control peptide for 18 h at 37°C in a 5% CO_2 incubator. To activate the JAK/STAT signaling pathway, U937 cells were then incubated in the presence or absence of 1000 U/mL IFNγ (PBL Biochemical Laboratories, Piscataway, NJ) for an additional 30 min. The media was aspirated out and the cells washed twice with cold PBS to remove media and cell debris. Cell lysates were prepared by adding 250 μL of cold lysis buffer (50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 50 mM NaF, 5 mM EDTA, and 0.1% NP40) containing protease inhibitor cocktail (Amersham Bioscience) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Lysis was allowed to proceed for 1 h at 4°C (rocking) to ensure complete cell lysis. Lysates were then centrifuged and supernatant transferred into fresh microcentrifuge tubes, protein concentrations determined, and protein lysates resolved by SDS-PAGE on a 12% polyacrylamide gel (Bio-Rad Laboratories). Proteins were transferred onto nitrocellulose membranes (Amersham Biosciences), placed in blocking buffer (5% nonfat dry milk and 0.1% Tween-20 in TBS), and washed in 0.1% Tween-20 in TBS. To detect phosphorylated STAT1α, membranes were incubated with pY701-STAT1α antibody (1:400 dilution; Santa Cruz) in blocking buffer for 2 h at room temperature. After four washes, the membranes were incubated in HRP-conjugated goat anti-rabbit IgG secondary antibody (1:2000 dilution; Santa Cruz) in blocking buffer for 1 h at room temperature, washed

four times, incubated for 1 min with ECL detection reagents (Amersham Biosciences) and exposed to photographic film (Amersham Biosciences) to visualize protein bands.

Macrophage Activity

Murine macrophage cells, Raw 264.7, were seeded on 24-well plates at a concentration of 3 x 10^5 cells/ well and allowed to adhere. Varying concentrations of the lipophilic peptides, Tkip, SOCS1-KIR or MuIFNγ(95-106), were then added to the wells and the cells incubated for 2 h at 37°C in a 5% CO_2 incubator. Recombinant IFNγ, in varying concentrations, was then added and the cells incubated for an additional 72 h at 37°C in a 5% CO_2 incubator, after which 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, San Diego, CA). To test for synergy between Tkip and SOCS1-KIR, the cells were incubated in the presence of IFNγ and varying concentrations of peptides as described above and also in the presence of both lipophilic Tkip and lipophilic SOCS1-KIR or lipophilic Tkip and lipophilic MuIFNγ(95-106). Supernatants were collected after 48 h and tested for nitric oxide production as described above.

Tkip Cellular Targets

SJL/J mice were immunized with bovine myelin basic protein (MBP) as previously described (Mujtaba et al. 2005). Briefly, 6 to 8 week old female SJL/J mice were immunized subcutaneously at two sites on the base of the tail, with MBP (300 μg/mouse) in complete Freund's adjuvant. At the time of MBP immunization and 48 h later, pertusis toxin (400 ng/mouse) was administered (i.p). This protocol was approved by IACUC at the University of Florida. The mice were observed daily for signs of EAE, and severity of disease was graded using the following scale: 1) loss of tail tone; 2) hind limb weakness; 3) paraparesis; 4)

paraplegia; and 5) moribund/death. The first physical signs of disease were generally observed beginning on day 18 to 21 after MBP immunization. Spleens were extracted after disease onset and homogenized into a single cell suspension. Splenocytes (1 x 10^5 cells/well) were incubated with medium, MBP (50 μg/mL), lipo-Tkip, lipo-SOCS1-KIR or control peptide lipo-MuIFNGR(253-287) for 48 h at 37°C in 5% CO_2. To test the effect of the peptides on cell proliferation, the cultures were pulsed with [^{3}H]-thymidine (1.0 μCi/well; Amersham Biosciences) for 18 h before harvesting onto filter paper discs using a cell harvester. Cell associated radioactivity was quantified using a beta scintillation counter and data are reported as counts per minute (cpm).

To test for the effect of Tkip on specific cellular targets, splenocytes obtained as described above were enriched for the desired cell type using negative isolation kits purchased from Dynal Biotech (Dynal Biotech, Oslo, Norway). The enrichments were carried out according to the manufacturer's instructions. Mouse B cells negative isolation kit, mouse $CD4^+$ T cells negative isolation kit, or mouse $CD8^+$ T cells negative isolation kit was used for B cells, $CD4^+$ T cells, or $CD8^+$ T cells, respectively. The use of the isolation kits results in cells enriched for the specific cell type, but to confirm the purity level, the cells may be stained with cell-type specific antibodies and FACS (fluorescence activated cell sorter) analysis carried out. This would have given a better indication of the actual purity level, and would provide an indication what contaminants (if any) were present.

Enriched cells were incubated with varying concentrations of appropriate lipophilic peptides in the presence or absence of antigen presenting cells (APCs), and in the presence or absence of antigen (MBP). The APCs were derived from splenocytes obtained from naïve SJL/J mice, which were incubated with MBP for 48 h, fixed with 2% paraformaldehyde for 30 min,

and washed extensively to remove residual paraformaldehyde. The cells were then transferred into mouse IFNγ-ELISPOT plates (Mabtech Inc, USA) and incubated for 48 h, after which the wells were washed, incubated with secondary antibody, washed, and spots developed according to manufacturer's instructions. The plates were then blotted dry, spots counted, and the data plotted using Graph Pad Prism 4.0 software (Graph Pad Software, San Diego, CA).

To test for the effect of Tkip on antibody production, B cells (5×10^5 cells/well) from MBP sensitized mice obtained two months after disease remission were incubated with varying concentrations of lipophilic peptides in the presence of MBP (50 μg/mL) and APCs and incubated for 48 h. Culture supernatants were then harvested and tested for MBP-specific antibodies by enzyme-linked immunoabsorbent assay (ELISA).

Antiviral Assays for SOCS-1 Antagonist Function

Antiviral activity was determined using the standard viral cytopathogenic effect assay described previously with minor modifications (Langford et al. 1981). Briefly, human fibroblast WISH cells at 70–80% confluency, were incubated in media alone or 0.4 U/mL human IFNγ (PBL Biomedical Laboratories) or both 0.4 U/mL human IFNγ and lipo-pJAK2(1001-1013) or lipo-JAK2(1001-1013) for 22 h in DMEM containing 2% FBS (maintenance media). Following incubation, WISH cells were washed once with maintenance media and infected with encephalomyocarditis virus (EMCV) (200 pfu/well) for 1 h at 37°C. The WISH cells were then washed once to remove unbound viral particles and incubated in fresh maintenance media for an additional 24 h at 37°C in a 5% CO_2 incubator. Plates were subsequently blotted dry and stained with 0.1% crystal violet solution for 5 min. Unbound crystal violet was aspirated and the plates thoroughly rinsed with deionized water, blotted, and air-dried. Viral plaques were counted using

a dissecting microscope and antiviral activity was determined by comparing experimental treatment groups with the virus only control group.

Transfections of LNCaP Cells with SOCS-1 DNA

Transfections were carried out to introduce SOCS-1 DNA into mammalian cells and test the ability of pJAK2(1001-1013) peptide to reverse SOCS-1 inhibition of STAT3 phosphorylation. Human prostate cancer cells, LNCaP, were plated in a 6-well plate and allowed to grow to 60% confluency. SOCS-1 plasmid DNA (1.6 μg/well) (pEF-FLAG-I/mSOCS1), a gift from Dr. David Hilton (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) or an empty vector, was transfected into the LNCaP cells using lipofectamine (Invitrogen Corporation, Carlsbad, CA) according to manufacturers instructions, but with modifications. The cells were incubated for 4 h, after which the transfection media was aspirated, fresh complete media (DMEM supplemented with 10% FBS and 100 U/mL streptomycin and 100 U/mL penicillin) added, and the cells incubated for an additional 72 h at 37°C. The complete media was then aspirated out, fresh media containing lipo-pJAK2(1001-1013) (20 μM) or control peptide, lipo-MuIFNγ(95-125), added to the transfected cells, and the JAK/STAT signaling pathway activated by adding IL-6 (50 ng/mL). Cells were incubated for 30 min prior to harvesting. Cell extracts were resolved by SDS-PAGE on a 12% polyacrylamide gel, transferred onto nitrocellulose membrane (Amersham Biosciences), and probed with phosphorylated (pY705) STAT3 antibody (Santa Cruz Biotechnology). The membranes were stripped and reprobed with unphosphorylated STAT3 antibody (Santa Cruz Biotechnology). Detection of proteins was accomplished using ECL detection reagents (Amersham Biosciences).

For immunoprecipitations, LNCaP cells growing on 60 mm plates and at 50% confluency were transfected with either SOCS-1 plasmid DNA (8 μg/plate) or empty vector (8 μg/plate) in

lipofectamine and incubated for 4 h after which 5 mL complete media was added and cells allowed to grow for 72 h. The cells were harvested in lysis buffer as described for western blot analysis, and the cell lysates centrifuged at 10000 x g to remove cellular debris and nuclei. Supernatants were transferred into fresh tubes and incubated with 2 μg/mL anti-Flag antibody (Sigma-Aldrich) for 2 h at 4ºC while rotating. Protein G PLUS-agarose beads (Santa Cruz Biotechnology) were added to the supernatants and allowed to incubate for 2 h at 4ºC while rotating, followed by centrifugation to pellet protein G immune complexes. Supernatants were discarded and the immune complexes washed three times with lysis buffer and once with PBS. The immune complexes were then heated (95ºC/5 min) in 50 μL of 1 X SDS sample buffer, resolved on a 12% polyacrylamide gel, transferred onto nitrocellulose, and immunoblotted with anti-SOCS-1 antibody (Santa Cruz Biotechnology). Detection of proteins was accomplished using ECL detection reagents (Amersham Biosciences).

GAS Promoter Activity

A plasmid, pGAS-Luc, that contains the promoter for IFNγ-activated sequence (GAS) linked to firefly luciferase gene was obtained from Statagene (La Jolla, CA). A constitutively expressed thymidine kinase promoter-driven *Renilla* luciferase gene (pRL-TK) (Promega Corporation, Madison, WI) was used as internal control in reporter gene transfections. WISH cells (1 x 10^5 cells/well) were seeded in a 12-well plate and incubated overnight at 37ºC, following which 3 μg GAS promoter-driven firefly luciferase expressing plasmid DNA and 10 ng pRL-TK were cotransfected into the WISH cells, using Fugene 6 (Roche Diagnostics Corporation, Indianapolis, IN). Two days later, the cell lysates were used to assay for firefly luciferase and *Renilla* luciferase, using a dual luciferase assay kit (Promega Corporation).

Luciferase activity, in relative luciferase units, was calculated by dividing firefly luciferase activity by *Renilla* luciferase activity in each sample.

Primer Design and PCR Amplification of Murine SOCS-1 DNA

Primers were designed to amply full-length SOCS-1 from the mouse SOCS-1 intracellular expression vector, pEF-FLAG-I/mSOCS-1 previously described. Primers were designed for compatibility with the multiple cloning site (MCS) of pBlueBac4.5/V5-His TOPO TA vector (Invitrogen). The primers were purchased from Integrated DNA Technology Inc and the primer sequences are shown below.

Forward primer 1

5'- AGG ATG GTA GCA CGC AAC CAG GT – 3'

Reverse primer

5'- GAT CTG GAA GGG GAA GGA AC – 3'

PCR reactions were carried out in 50 μL reactions containing dNTPs (0.2 mM each), $MgCl_2$ (1.5 mM), primers (0.2 μM each), template DNA (1 μg), platinum *Taq* DNA polymerase (1 unit), platinum *Pfx* polymerase (1 unit), 10 X PCR buffer (5 μL) and DNAse free water. The thermocycler was programmed for 30 cycles at 94^{o}C for 30 sec, 50^{o}C for 30 sec, 72^{o}C for 1 min, with initial denaturation at 94^{o}C for 4 min and final extension at 72^{o}C for 7 min. The PCR products were resolved on a 1% TAE agarose gel, stained with ethidium bromide, and photographed.

Cloning SOCS-1 into pBlueBac4.5/V5-His TOPO TA Expression Vector

The PCR products of correct size (650 bp) were excised out of the gel, purified using Wizard PCR prep DNA purification system (Promega, Madison WI) and cloned into the pBlueBac4.5/V5-His TOPO TA expression vector according to the manufacturer's instructions (Invitrogen). Following cloning, the recombinant vector was used to transform TOP10

chemically competent cells according to manufacturer's instructions (Invitrogen), and the transformed cells grown on LB-ampicilin nutrient agar plates overnight at 37^0C. Positive and negative control cloning reactions were carried out similar to those for SOCS-1 DNA, but in the absence of SOCS-1 DNA. Positive clones were identified by PCR, and plasmid DNA isolated from the PCR positive clones. Restriction enzyme digestion and DNA sequencing were used to confirm the presence of SOCS-1 DNA.

Expression of SOCS-1 in Sf9 Cells

The recombinant pBlueBac4.5/His-V5 TOPO vector carrying the murine SOCS-1 gene (pBlueBac4.5/muSOCS-1) was transfected into the expression vector Bac-N-Blue (Invitrogen) according to the manufacturer's instructions. Briefly, the Bac-N-Blue vector contains a triple-cut, linearized AcMNPV (*Autographa californica* multiple nuclear polyhedrosis virus). The linearized virus lacks sequences essential for efficient propagation, specifically sequences in the ORF1629. Hence, for successful propagation and isolation of viable virus, the essential ORF1629 sequences need to be supplied by a transfer vector. The pBlueBac4.5/His-V5 TOPO vector contains these essential sequences. The pBlueBac/muSOCS-1 and Bac-N-Blue were co-transfected into Sf9 (*Spodoptera frugiperda*) insect cells growing at 50% confluency (Invitrogen) in the presence of cellfectin reagent (Invitrogen) in unsupplemented Grace Insect Medium (Invitrogen). The transfection was carried out at room temperature and the transfection complexes incubated with the cells for 6 h following which complete TNM-FH medium was added and the cells allowed to grow for an additional 72 h. Half the volume of the culture media was harvested and used for plaque assay. The same volume of fresh TNM-FH media was added to the cells, and the cells allowed to grow for an additional 72 h at which point significant cell lysis was observed. The virus (P1 stock) was then collected by centrifugation and tested for the presence of murine SOCS-1 DNA by PCR using the Baculovirus primers.

Forward primer 5'- TTTACTGTTTTCGTAACAGTTTTG - 3'

Reverse primer 5'– CAACAACGCACAGAATCTAGC – 3'

In order to generate high titer recombinant virus, PCR positive clones were propagated further and used to infect fresh Sf9 cells growing in suspension culture. The cells were allowed to grow and harvested at different time points to determine the best time for harvesting cells expressing SOCS-1 protein. It was determined that 96 h infection provided the highest SOCS-1 protein yield, and subsequently Sf9 cells were harvested 96 h post infection. Cell lysates were harvested using native conditions as described in the ProBond purification manual (Invitrogen) and the expression of murine SOCS-1 confirmed by immunoblot analysis using anti-SOCS-1 antibody (Santa Cruz).

Statistical Analysis

The data were not normally distributed therefore nonparametric statistical analyses tests, the Mann-Whitney and Wilcoxon tests were used. The Mann-Whitney signed rank sum test compares two groups and performs calculations on the rank of the values, rather than the actual data (Motulsky 1995). It is considered to be similar to the t-test, except the data are not normally distributed. The Wilcoxon signed rank sum test compares two paired groups of nonparametric data (Motulsky 1995). It is similar to the paired t-test, but the data are not normally distributed. All calculations were performed using the GraphPad Prism statistical package (GraphPad Software Inc, San Diego, CA).

Table 3-1. List of peptides used in this study. DTkip and DRTkip were synthesized from D-amino acids. All the other peptides were synthesized from L-amino acids. The Y1007 in pJAK2(1001-1013), in italics, is phosphorylated.

Peptide	Sequences
Tkip	WLVFFVIFYFFR
DTkip	WLVFFVIFYFFR
DRTkip	RFFYFIVFFVLW
JAK2(1001-1013)	^{1001}LPQDKEYYKVKEP
pJAK2(1001-1013)	^{1001}LPQDKE*Y*YKVKEP
MuIFNγ(95-106)	^{95}AKFEVNNPQVQR
MuIFNγ(95-125)	^{95}AKFEVNNPQVQRQAFNELIRVVHQLLPESSL
SOCS1-KIR	^{53}DTHFRTFRSHSDYRRI
SOCS1-ESS	^{68}ITRASALLDACG
MuIFNGR1(253-287)	^{253}TKKNSFKRKSIMLPKSLLSVVKSATLETKPESKYS

MuIFNγ(95-106), MuIFNγ(95-125), and MuIFNR1(253-287) were used as control peptides. These peptides do not show significant biological activity in the assays for which they have been used as control peptides.

CHAPTER 4
RESULTS

Tkip Family Members Bind to JAK2 Autophosphorylation Site

It has previously been shown that Tkip binds to the autophosphorylation site of JAK2 and inhibits JAK2 autophosphorylation and phosphorylation of IFNGR-1 (Flowers et al. 2004). Here I show that DTkip and DRTkip but not the control peptide, MuIFNγ(95-106), bound to the JAK2(1001-1013) peptide in a dose-dependent manner (Figure 4-1a). Thus, these SOCS-1 mimetic peptides bind JAK2 autophosphorylation site peptide in a dose-dependent manner.

Current literature suggests that phosphorylation of JAK2 tyrosine 1007 is important in SOCS-1 mediated JAK2 ubiquitin-proteosome-dependent degradation (Ungureanu et al. 2002). Hence, I wanted to determine whether these peptides, like SOCS-1, bind to phosphorylated JAK2, pJAK2(1001-1013). Dose response solid-phase ELISA were carried out with biotinylated pJAK2(1001-1013) peptide in place of biotinylated JAK2(1001-1013) peptide. DTkip and DRTkip bound with two to threefold greater affinity to pJAK2(1001-1013) than to JAK2(1001-1013) as shown in Figure 4-1b. This is consistent with previous data showing that Tkip binds with a greater affinity to pJAK2(1001-1013) than to JAK2(1001-1013) (Flowers et al. 2004). The binding observed was dose dependent, implying specificity in binding. Hence, the SOCS-1 mimetic peptides recognize JAK2 autophosphorylation site similar to Tkip, implication of which is that they recognize JAK2 autophosphorylation similar to SOCS-1.

In order to determine whether Tkip and DRTkip bind to the same site on JAK2 autophosphorylation site, competition for binding assays were carried out. Soluble Tkip and soluble DRTkip, but not the control peptide (MuIFNγ95–106) inhibited binding of biotinylated JAK2(1001-1013) to immobilized Tkip in a dose dependent manner (Figure 4-2a)**.** Next, the competition for binding of Tkip with DRTkip to pJAK2(1001-1013) was determined. As shown

in Figure 4-2b, Tkip and DRTkip inhibited the binding of pJAK2(1001-1013) to immobilized Tkip. Hence, Tkip and DRTkip likely bind to the same site on JAK2 autophosphorylation site, the implication of which is that DRTkip may have similar binding characteristics as Tkip to JAK2. The D amino acid isomer thus, binds similar to the L isomer, suggesting a stable form of Tkip for functional studies.

Tkip Family Members Inhibit JAK2 Kinase Activity

SOCS-1 regulates JAK2 activity by interacting with the autophosphorylation site and inhibiting JAK2 kinase activity. Therefore, DRTkip was tested for its ability to inhibit JAK2 autophosphorylation and JAK2 phosphorylation of substrate (IFNGR-1). DRTkip but not the control peptide, MuIFNγ(95-106) inhibited JAK2 autophosphorylation and JAK2 induced phosphorylation of IFNGR-1 (Figure 4-3). These results are similar to what has been previously shown with Tkip (Flowers et al. 2004). The data suggest that DRTkip may inhibit both JAK2 activation and JAK2-mediated phosphorylation of IFNGR-1, the implication of which is that the SOCS-1 mimetic peptides, like SOCS-1, may potentially regulate IFNγ signaling. It is worth noting that like Tkip, DRTkip did not inhibit tyrosine phosphorylation of c-Src kinase (data not shown), which is consistent with the fact that c-Src kinase is not inhibited by SOCS-1.

Tkip Inhibits Superantigen-induced Proliferation of Mouse Splenocytes

The staphylococcus superantigens are potent T cell mitogens that exert their effects by forming complexes with MHC class II molecules on antigen presenting cells, and binding to the T cell receptor (TCR) via the V_{β}-element of the (TCR), resulting in activation of the T cells (Reviewed in Torres et al. 2001). Our laboratory has shown that superantigens such as staphylococcus entrotoxin A and B (SEA and SEB) can exacerbate immunological disease and induce relapses in the mouse model for multiple sclerosis, experimental allergic encephalomyelitis, EAE, (Torres et al. 2001). It has previously shown that Tkip inhibits antigen-

induced proliferation of splenocytes (Mujtaba et al. 2005). Here the ability of Tkip and DRTkip to inhibit superantigen-induced proliferation of primary cells was tested. Mouse splenocytes were stimulated with SEB (500 ng/mL) in the presence of lipophilic Tkip, lipophilic DRTkip, or lipophilic control peptide, MuIFNγ(95-106) (a lipophilic group, lysyl-palmitate, is added to the N-terminal ends of the peptides to facilitate entry into the cells) and incubated for 72 h prior to pulsing with ^{3}[H]-thymidine. As shown in Figure 4-4a, Tkip but not DRTkip inhibited SEB induced proliferation of the splenocytes. Next I tested the ability of Tkip and DRTkip to inhibit SEA-induced splenocytes proliferation. Tkip, but not DRTkip inhibited SEA-induced splenocytes proliferation (Figure 4-4b). Next the ability of Tkip and DRTkip to inhibit STAT1α phosphorylation in murine fibroblast cells (U937) was tested. Tkip, but not DRTkip inhibited STAT1α phosphorylation (Data not shown). The lysyl-palmitate group is an L-lysine and may be affected by proteases in such a way as to affect the efficiency of DRTkip uptake by cells. It is also possible that the D-isomer amino acids may result in a peptide whose conformation is different enough from Tkip to have slightly different cellular function. However, since only a limited number of cellular function assays were carried out, it can not be conclusively be determined whether DRTkip has or does not have intracellular SOCS-1 like function. Thus, factors currently unknown may affect DRTkip intracellular function.

The observations that DRTkip, unlike Tkip, did not seem to significantly affect biological activity suggested that the use of the Tkip retro-inversion, DRTkip, may have changed the orientation of the amino acids enough to affect function. Hence, this research focused on Tkip and SOCS1-KIR and not the other SOCS-1 mimetics. There is however, continued interest in why DRTkip is not the same as Tkip in cell functional comparisons.

SOCS-1 Kinase Inhibitory Region (SOCS1-KIR) Binds to JAK2 Autophosphorylation Site

Yasukawa et al. (1999) suggested that the binding of SOCS-1 to JAK2 requires the SOCS-1 SH2-domain and the extended SH2 domain (ESS) and that the kinase inhibitory region (KIR), while not required for the initial binding is essential for the inhibitory action of SOCS-1. Here, I present results showing that a peptide corresponding to SOCS-1 KIR region, SOCS1-KIR, specifically binds to a peptide representing the JAK2 autophosphorylation site and inhibits STAT1α activation. Further, I show that SOCS1-KIR, as well as Tkip, inhibit IFNγ-induced macrophage activation. In addition, I show that a peptide corresponding to the ESS region, SOCS1-ESS. Does not bind to the JAK2 autophosphorylation site, the implication of which is that the SH2, ESS, and KIR regions may all play role in the binding of SOCS-1 to JAK2.

Tkip and SOCS1-KIR Bind to JAK2 Autophosphorylation Site

First I determined whether SOCS1-KIR, like the SOCS-1 mimetic Tkip, binds to the JAK2 autophosphorylation site by carrying out dose-response solid-phase binding assays with JAK2 autophosphorylation site peptide, JAK2(1001-1013). Tkip, SOCS1-KIR, or a control peptide, MuIFNγ(95-106), were immobilized on 96-well microtiter plates and incubated with biotinylated JAK2(1001-1013) at various concentrations. Tkip and SOCS1-KIR, but not the control peptide, bound to the JAK2(1001-1013) peptide in a dose-dependent manner (Figure 4-5a). Thus, both Tkip and SOCS-1-KIR specifically bind to the JAK2 autophosphorylation site peptide. While this is consistent with the SOCS-1 mimetic character of Tkip, it also provides direct evidence that the KIR region of SOCS-1 can interact directly with JAK2 autophosphorylation site, suggesting that Tkip and KIR recognized a similar site on JAK2.

Since phosphorylation of Y1007 is required for high catalytic activity of JAK2, it is logical that SOCS-1 would bind with higher affinity to Y1007 phosphorylated JAK2. Consistent with

this, it has previously been shown that Tkip binds with greater affinity to Y1007 phosphorylated JAK2(1001–1013) peptide, pJAK2(1001-1013), than to unphosphorylated JAK2 peptide (Flowers et al. 2004). I therefore determined whether SOCS1-KIR bound to pJAK2(1001–1013) with greater affinity than to JAK2(1001–1013). Both SOCS1-KIR and Tkip bound to pJAK2(1001-1013) with two to three-fold greater affinity than to JAK2(1001-1013) as shown in Figure 4-5b. In addition, SOCS1-KIR bound to pJAK2(1001-1013) with higher affinity than Tkip. Thus, SOCS1-KIR recognizes JAK2 autophosphorylation similar to Tkip, the implication of which is that Tkip recognizes the JAK2 autophosphorylation site similar to SOCS-1.

To determine whether Tkip and SOCS1-KIR bind to the same site on the JAK2 autophosphorylation site, binding competition assays were carried out. Tkip or SOCS1-KIR was immobilized on a 96-well plate and biotinylated JAK2(1001-1013) or biotinylated pJAK2(1001-1013), which had been pre-incubated with Tkip, SOCS1-KIR or a control peptide, was allowed to bind to the immobilized peptides. As shown in Figure 4-6a, soluble Tkip and soluble SOCS1-KIR, but not soluble control peptide, inhibited the binding of biotinylated JAK2(1001-1013) to immobilized Tkip. A similar pattern of inhibition was observed with biotinylated JAK2(1001-1013) binding to immobilized SOCS1-KIR (Figure 4-6b). Homologous inhibition was slightly better for both Tkip and SOCS1-KIR, which suggests slight differences in recognition of JAK2 autophosphorylation site.

Next, the binding competition of Tkip and SOCS1-KIR to pJAK2(1001-1013) was determined. The competition for binding to pJAK2(1001-1013) was similar to that observed in competition for binding to unphosphorylated JAK2 peptide (Figure 4-6c and 4-6d). Again, homologous competition was slightly better, which again suggests slight differences in recognition of the JAK2 autophosphorylation site. These data provide direct evidence that the

mimetic effect of Tkip is applicable to the KIR region of SOCS-1 and that Tkip and KIR recognize JAK2 autophosphorylation site similar but not exactly the same.

JAK2 Kinase Activity and STAT1α Activation

SOCS-1 regulates JAK2 activity at least at two levels. One involves interaction with the autophosphorylation site, which affects JAK2 phosphorylation of substrates such as STAT1 and STAT3 (Yasukawa et al. 1999). The other level involves induction of proteosomal degradation of both JAK2 and SOCS-1, requiring the SOCS box domain of SOCS-1 (Zhang et al. 2001). Obviously, neither Tkip nor SOCS1-KIR has a SOCS box, so the two peptides were compared for their relative ability to inhibit JAK2 autophosphorylation as well as phosphorylation of the transcription factor, STAT1. As shown in Figure 4-7a**,** Tkip but not SOCS1-KIR inhibited JAK2 autophosphorylation. This would suggest that the similar but slight differences in recognition of JAK2 resulted in significant differences in regulation of JAK2 autophosphorylation.

Next the two peptides were compared for their relative ability to inhibit IFNγ activation of STAT1α in murine U937 cells. In contrast to inhibition of JAK2 autophosphorylation, both Tkip and SOCS1-KIR inhibited JAK2 mediated phosphorylation of STAT1α (Figure 4-7b). Thus, Tkip inhibits JAK2 autophosphorylation as well as JAK2 mediated phosphorylation of STAT1α in murine U937 cells, while SOCS1-KIR does not inhibit JAK2 autophosphorylation, but does inhibit JAK2 mediated phosphorylation of STAT1α transcription factor. SOCS1-KIR thus shows the same regulatory pattern as SOCS-1, in that JAK2 autophosphorylation is not inhibited, while STAT1α substrate phosphorylation by activated JAK2 is inhibited. It has previously showed that Tkip, like SOCS-1, also inhibited EGFR autophosphorylation (Flowers et al. 2004). Thus SOCS1-KIR was tested for ability to inhibit EGFR phosphorylation. As shown in Figure 4-7c**,**

both peptides inhibited EGFR phosphorylation with Tkip being the more effective inhibitor. SOCS1-KIR is thus similar to SOCS-1 in its kinase inhibitory function.

Tkip and SOCS1-KIR Inhibit IFNγ-induced Activation of Macrophages

IFNγ plays an important role in activation of macrophages for innate host defense against intracellular pathogens as well as serving to bridge the link between innate and adaptive immune responses (Reviewed in Boehn et al. 1997). Thus, Tkip and SOCS1-KIR were examined for their ability to block IFNγ activation of the murine macrophage cell line Raw 264.7 as determined by inhibition of nitric oxide (NO) production using Griess reagent (Alexis Biochemicals). Lipophilic (lipo) versions of the peptides were synthesized with palmitic acid for penetration of the cell membrane (Thiam et al. 1999). Both Tkip and SOCS-KIR, compared to control peptide, MuIFNγ(95-106), inhibited induction of NO by various concentrations of IFNγ as shown in Figure 4-8a. Dose-response with varying concentrations of the peptides against IFNγ (6 U/mL) resulted in increased inhibition of NO production by Tkip and SOCS1-KIR with Tkip being the more effective of the inhibitors as shown in Figure 4-8b. The control peptide, MuIFNGR1(253-287) was relatively ineffective at inhibition, providing evidence for the specificity of Tkip and SOCS1-KIR inhibition. Tkip and SOCS1-KIR in combination (33 μM each) were the most effective in inhibition of IFNγ induction of NO in macrophages. This synergy may reflect differences in recognition of the autophosphorylation site of JAK2 by the two peptides. Thus, Tkip and SOCS1-KIR both inhibited IFNγ induction of NO in macrophages with Tkip being the more effective inhibitor.

Tkip and SOCS1-KIR Inhibit Antigen-specific Lymphocyte Proliferation

Our laboratory has previously shown that Tkip inhibits antigen-specific proliferation of mouse splenocytes *in vitro* (Mujtaba et al. 2005). Specifically, Tkip inhibited proliferation of

splenocytes from mice immunized with bovine myelin basic protein (MBP). Here, I compared Tkip and SOCS1-KIR for their relative ability to inhibit proliferation of MBP-specific splenocytes in cell culture. Splenocytes (3×10^5 cells/well) were incubated with MBP (50 μg/mL) in the presence of lipo-Tkip, lipo-SOCS1-KIR, or lipo-control peptide for 48 h and proliferation assessed by testing for [^{3}H]-thymidine incorporation. As shown in Figure 4-9, both Tkip and SOCS1-KIR inhibited MBP-induced proliferation of splenocytes, while the control peptide had a negligible effect on proliferation. Similar to inhibition of NO production by macrophages, Tkip was more effective than SOCS1-KIR in inhibition of MBP induced splenocyte proliferation with 84, 88, and 97% inhibition at 1.2, 3.7, and 11 μM, respectively, compared to 61, 67, and 72% for SOCS1-KIR. Thus, both Tkip and SOCS1-KIR inhibited antigen-induced splenocyte proliferation, which is consistent with SOCS-1 protein inhibition of antigen-specific lymphocyte activity (Cornish et al. 2003).

An Extended SH2 Sequence (SOCS1-ESS) Peptide does not Bind to pJAK2 (1001-1013)

Based in part on binding experiments with truncations of SOCS-1 protein, it has been proposed that the SH2 domain plus ESS bind to JAK2 at the activation site represented by peptide pJAK2(1001-1013), while SOCS1-KIR binds primarily to the catalytic site of JAK2 (Yasukawa et al. 1999). Therefore the SOCS1-ESS peptide, ^{68}ITRASALLDACG, was synthesized and compared with SOCS1-KIR for binding to biotinylated pJAK2(1001-1013).

As shown in Figure 4-10**,** SOCS1-KIR as well as Tkip bound biotinylated pJAK2(1001-1013) in a dose-response manner, while SOCS1-ESS failed to bind. Hence, the SOCS1-KIR peptide (residues 56–68), but not the SOCS1-ESS peptide (68–79) binds to the JAK2 autophosphorylation site. The SOCS1-KIR peptide, except for residues 53-55 is contained in the SOCS-1 ESS-SH2 construct, dN56, (Yasukawa et al. 1999) that bound to JAK2

autophosphorylation site. dN56 is a construct in which sequences N-terminal to amino acid residue 56 have been truncated, therefore it contains KIR, ESS, SH2 domain, SOCS box, and the C-terminal sequences. Thus, SOCS1-KIR, which is N-terminal and contiguous with SOCS1-ESS, probably shares overlapping functional sites with SOCS1-ESS. Clearly, SOCS1-KIR is preferentially recognized by JAK2(1001-1013) compared to the 12-mer SOCS1-ESS. The specific role of the various residues in the SH2 domain of SOCS-1 in JAK2 and pJAK2(1001-1013) binding remains to be determined.

Tkip Cellular Targets

Studies carried out in our laboratory have shown that Tkip affects the growth of cells growing in culture, as well as the progression of EAE. Specifically, Tkip inhibits LNCaP and DU145 prostate cancer cells proliferation and inhibits antigen-specific cell proliferation Tkip at 63 μg/mouse, given every other day prevented development of acute form of EAE, and induced stable remission in the chronic relapsing/remitting form of EAE (Flowers et al. 2004, Flowers et al. 2005, Mujtaba et al. 2005). Moreover, no toxicity was observed when Tkip at 200 μg/mouse, given every other day for one week (Mujtaba et al. 2005). These data suggested that Tkip may have direct effect on cells of the immune system. Hence, I attempted to define Tkip cellular targets. Data are presented showing that Tkip specifically targets antigen presenting cells (APCs), $CD4^+$ T, $CD8^+$ T, and B cells. In addition preliminary data on SOCS1-KIR peptide cellular targets are also presented.

Effect of Tkip and SOCS1-KIR on $CD4^+$ T Cells

In order to identify Tkip cellular targets, I first asked whether Tkip had any effect on primary splenocytes derived from myelin basic protein (MBP) sensitized mice. Sensitization of mice with MBP, in the presence of adjuvant, results in the development of experimental allergic encephalomyelitis, EAE, a disease characterized by paralysis. As shown in Figure 4-9, Tkip and

SOCS1-KIR inhibit antigen-specific primary cell (splenocytes) proliferation in a dose-dependent manner. Next I asked, which cell populations were targeted by Tkip. First the effect of Tkip and SOCS1-KIR on $CD4^+$ T cell subset was tested. $CD4^+$ T cells are important in generating effective cell mediated immunity and in mediating humoral immune responses. $CD4^+$ T cells obtained from MBP sensitized mice after disease onset were incubated with lipophilic peptides, in the presence or absence of APCs and MBP. Both Tkip and SOCS1-KIR (33 μM), but not the control peptide, MuIFNGR1(253-287) inhibited $CD4^+$ T cell proliferation (Figure 4-11a). In addition, Tkip and SOCS1-KIR (33 μM), inhibited antigen-induced IFNγ production by $CD4^+$ T as determined by IFNγ ELISPOT assays (Figure 4-11b). The presence of MBP enhanced cell proliferation and the number of IFNγ-producing cells, but the presence of APCs did not seem to have such effects. The data presented for peptides were obtained in the presence of both MBP and APCs. Hence, Tkip and SOCS1-KIR target $CD4^+$ T cells and inhibit antigen-specific $CD4^+$ T cell proliferation and IFNγ production.

Effects of Tkip and SOCS1-KIR on $CD8^+$ T Cells

$CD8^+$ T cells play important roles in the immune response mechanism including cytotoxic T cell activity and production of cytokines that serve as effectors for various other immune responses. Since Tkip and SOCS1-KIR inhibited $CD4^+$ T cell activity, I asked whether the peptides could also target $CD8^+$ T cells. $CD8^+$ T cells derived from MBP-sensitized mice that were showing signs of active disease were treated with lipophilic peptides in the presence or absence of MBP and APCs. IFNγ ELISPOT assays carried out. Both Tkip and SOCS1-KIR, but not the control peptide, MuIFNGR1(253-287) showed a general trend in inhibiting MBP-induced IFNγ-production by $CD8^+$ T cells (Figure 4-12). The presence of MBP lead to a slight increase in the number of IFNγ-producing cells, but the presence of APCs did not seem to have such an effect. The data presented for peptides was obtained in the presence of both MBP and APCs.

Effect of Tkip and SOCS1-KIR on B Cells

B cells also play an important role in immune response mechanism. Activation of B cells results in B cell proliferation and differentiation into antibody producing cells and memory cells. B cells are also APCs. Tkip was tested for effect on B cells derived from MBP-sensitized mice, two months after disease remission. The B cells were treated with either lipophilic Tkip, lipophilic SOCS1-KIR, or lipophilic control peptide, MuIFNGR1(253-287), or no peptide, in the presence APCs and in the presence, or absence of unprocessed antigen (MBP). Tkip and SOCS1-KIR, in the presence of MBP and APCs, inhibit antigen-specific B cell proliferation (Figure 4-13a). In addition, both Tkip and SOCS1-KIR inhibit the secretion of MBP-specific antibodies in MBP-treated B cells (Figure 4-13b). The addition of APCs did not significantly affect the experimental results. The data shows that Tkip and SOCS1-KIR target B cell and inhibit both B cell proliferation and secretion of antibodies by plasma cells, the implication of which is that Tkip and SOCS1-KIR may have an effect on B cell activity. This is of specific interest in the study of EAE and multiple sclerosis, because the presence of MBP-specific antibodies and of inflammatory cytokines such as IFNγ has been shown to exacerbate disease. Hence, this may be one of the mechanisms by which Tkip inhibits disease progression in MBP-sensitized mice.

Effect of Tkip on Macrophages

I have described experiments showing that Tkip and SOCS1-KIR inhibit IFNγ-induced macrophage activation (Figure 4-8). I also tested the ability of Tkip to inhibit LPS-induced macrophage activity. Both Tkip and SOCS1-KIR inhibited LPS-induced macrophage activity (Figure 4-14). LPS signaling utilizes the Toll Like Receptor 4 (TLR4), a major pathway used in immune response to gram-negative bacteria. Hence, these results have possible implication on the effect that Tkip on TLR4 signaling and therefore in inhibition of excessive signaling through the TLR4. Such excessive signaling has been implicated in the development of inflammation.

Additional research is being carried out to determine additional effects that Tkip may have on TLR4 signaling. Since macrophages are APCs, these data are preliminary evidence that Tkip and SOCS1-KIR target APCs.

I have therefore shown that Tkip and SOCS1-KIR target and affect the activity of immune cells including $CD4^+$ T cells, $CD8^+$ T cells, B cells, and APCs, the implication of which Tkip and SOCS1-KIR may have effects on antigen-induce immune responses.

SOCS-1 Antagonist Activity of pJAK2 (1001–1013) Peptide

The demonstration that the KIR region of SOCS-1 could bind to the autophosphorylated JAK2 peptide raised the possibility that the phosphorylated peptide, pJAK2 (1001–1013), may inhibit the function of endogenous SOCS-1 and thus enhance IFNγ and IL-6 activities that are mediated by JAK2. As shown in Figure 4-15a, the antiviral activity of a suboptimal dose of IFNγ (0.4 U/mL) was enhanced against encephalomyocarditis virus (EMCV) in WISH cells by pJAK2(1001–1013). Specifically, unphosphorylated JAK2(1001–1013) at 11 μM final concentration reduced EMCV plaques relative to IFNγ alone by 42%, while the same concentration of pJAK2(1001–1013) reduced plaques by 59%. This is consistent with better binding of pJAK2(1001–1013) by SOCS1-KIR as shown above and by previous studies showing that SOCS-1 is active against JAK2 phosphorylated at tyrosine 1007 (Yasukawa et al. 1999). The peptide alone had no effect on EMCV, similar to media. Thus, pJAK2(1001–1013) boosts the activity of a suboptimal concentration of IFNγ, possibly interfering with endogenous SOCS-1 activity.

At the level of signal transduction, I examined the effects of pJAK2(1001–1013) on activation of STAT3 transcription factor in the LNCaP prostrate cancer cell line. The cells were treated with IL-6 to activate STAT3 signaling, which occurs through JAK2 kinase (Flowers et al.

2005). The SOCS-1 gene was overexpressed in these cells, which resulted in reduction of the level of IL-6 induced activation of pSTAT3 as shown in Figure 4-15b. Treatment of the cells with pJAK2(1001–1013) (20 μM) resulted in approximately a two-fold increase in activated STAT3 compared to IL-6 treated cells that were transfected with SOCS-1, as per densitometry readings. Expression of SOCS-1 protein in LNCaP cells is shown in Figure 4-15b. Thus, pJAK2(1001–1013) has an inhibitory effect on SOCS-1 at the level of signal transduction.

I next determined if pJAK2(1001–1013) could enhance GAS promoter activity of IFNγ. Accordingly, a plasmid with the GAS promoter element linked to the firefly luciferase reporter gene was cotransfected along with *Renilla* luciferase reporter plasmid as a control, into human WISH cells. As shown in Figure 4-16c, treatment of the WISH cells with IFNγ (1 U/mL) resulted in a four-fold relative increase in luciferase activity, which was increased to ten-fold and five-fold by 5 and 1 μM pJAK2(1001–1013), respectively. pJAK2(1001-1013) alone did not activate reporter gene (data not shown) and control peptide did not enhance IFNγ activation of reporter gene. Thus, consistent with the anti-SOCS-1 effects of pJAK2(1001–1013), the peptide also enhanced IFNγ function at the level of gene activation. It has recently been shown that suppression of SOCS-1 in dendritic cells by siRNA enhanced anti-tumor immunity (Shen et al. 2004). In order to determine the effect of pJAK2(1001-1013) on cell-mediated immune response, C56BL/6 mice were treated with pJAK2(1001-1013), control peptide, or PBS, following immunization with BSA. It was shown that pJAK2(1001-1013) enhanced BSA-induced proliferation of splenocytes by four to five fold when compared to control peptide or PBS (Waiboci et al. 2007). Further, I showed that supernatants containing SOCS-1 protein competed with SOCS1-KIR for binding to pJAK2(1001-1013) (Figure 4-15d). The demonstration of SOCS-1 competition for pJAK2(1001-1013) is consistent with pJAK2(1001-1013) antagonism

via sequestration of critical functional site(s) on SOCS-1. Hence, the data have shown that pJAK2(1001-1013) antagonizes SOCS-1 activity at five different levels; IFNγ antiviral function, IL-6 signal transduction, IFNγ activation of reporter gene via the GAS promoter, enhancement of antigen-specific proliferation, and possible sequestration of binding sites on SOCS-1.

Expression of SOCS-1 Protein

SOCS-1 is a negative regulator of immune factors including IFNs, IL-2, IL-4, and IL-6. SOCS-1 also modulates signaling by a variety of hormones. However, in spite of the presence of SOCS-1 and other immune modulators, the host defense system can pathologically perpetuate inflammation by overproducing immune mediators, such as inflammatory cytokines, that cause damage to multiple organs, resulting in what are referred to as inflammatory diseases/disorders. Further, it is estimated that approximately 20% of human cancers result from chronic inflammation. In addition, silencing of the SOCS-1 gene, by methylation, has been found in several human cancers (Hanada et al. 2006). Research carried our in our laboratory has shown that Tkip protects MBP sensitized mice from developing EAE, an inflammatory disease. Further, it has shown that Tkip inhibits proliferation of prostate cancer cells. Since Tkip is a SOCS-1 mimetic, I reasoned that recombinant SOCS-1 protein may have the same effect as Tkip in preventing inflammation and inhibiting proliferation of cancer cells. Therefore, recombinant SOCS-1 protein was expressed with the aim of obtaining protein to first characterize SOCS-1 functional sites and second to determine functional relationship to Tkip. The recombinant SOCS-1 protein was tested for binding to JAK2 autophosphorylation site.

First, primers were designed to amplify murine SOCS-1 (muSOCS-1) from an expression library, pEF-FLAG-I/mSOCS-1, a gift from Dr. D. Hilton (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). SOCS-1 DNA was amplified from the expression library, gel purified, and cloned into pBlueBac4.5/V5-His TOPO TA vector (Invitrogen). Plasmid DNA

was isolated and the presence of muSOCS-1 confirmed by restriction enzyme digestion (Figure 4-16a) and DNA sequencing (Figure 4-16b). I refer to the cloned product as pBlueBac/muSOCS-1. The constructs were co-transfected, with the baculovirus vector Bac-N-Blue (Invitrogen), into Sf9 insect cells and propagated. Co-transfection of the pBlueBac4.5/His-V5 TOPO vector carrying muSOCS-1, with Bac-N-Blue vector ensured that generally, only recombinant virus would be viable and therefore, would grow. Following transfection and propagation, viral plaques were picked and tested for presence of muSOCS-1 by PCR. As shown in Figure 4-17, several plaques had viral DNA of the correct size (650 bp). Of these, two were purified and used for protein expression. Western blot analysis of the expression of SOCS-1 protein in the Sf9 cells is shown in Figure 4-15d. The SOCS-1 lysates and the SOCS1-KIR peptide were used for competition for binding assays to test for ability to inhibit binding of pJAK2(1001-1013) peptide to immobilized SOCS1-KIR. Figure 4-15d, shows that SOCS1-KIR and SOCS-1 lysate, similar to Tkip and SOCS1-KIR competed for binding sites on pJAK2(1001-1013), the implications of which is that SOCS1-lysate may have characteristics similar to Tkip. The goal of the laboratory is to obtain sufficient purified SOCS-1 protein for detailed characterization of functional sites.

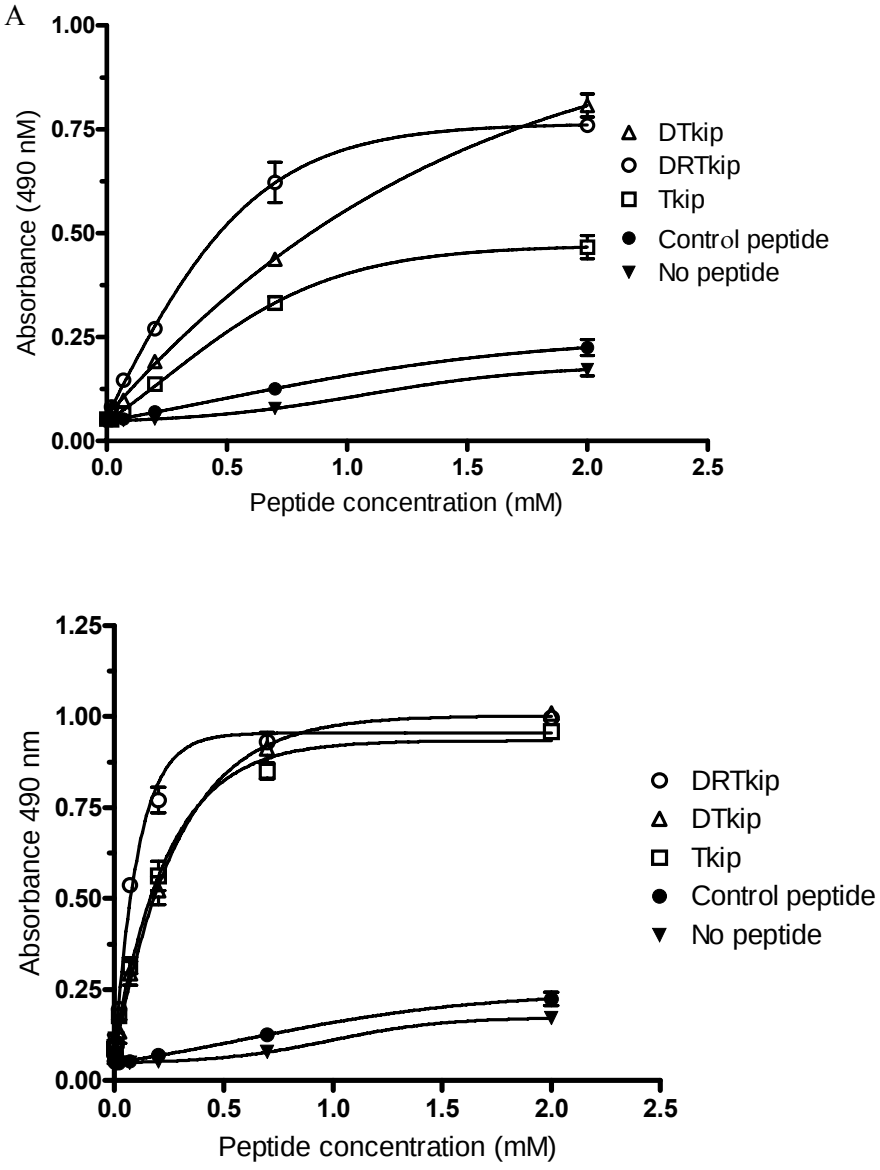
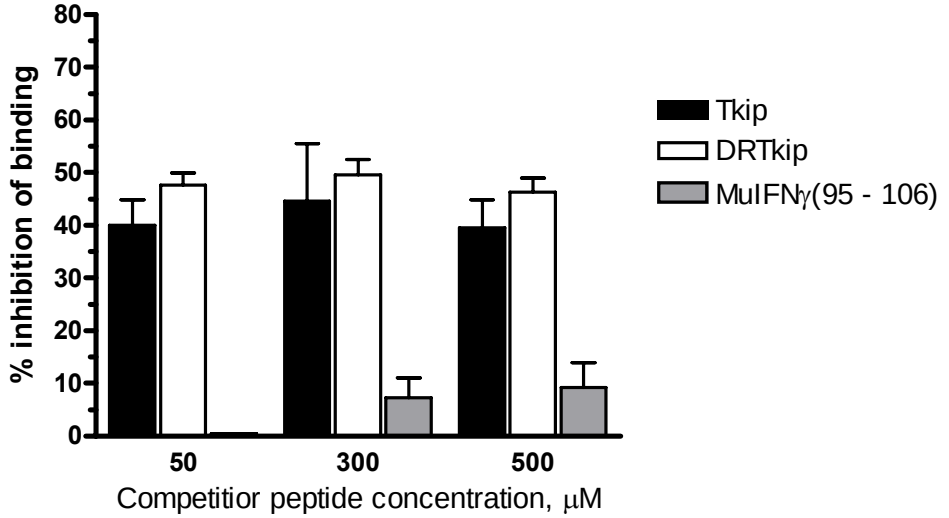


Figure 4-1. JAK2 autophosphorylation site peptides JAK2(1001-1013) and pJAK2(1001-1013) bind to SOCS-1 mimetic peptides. A) JAK2(1001-1013) peptide binds to Tkip, DTkip, and DRTkip. Biotinylated JAK2(1001-1013), at the indicated concentrations, was added in triplicate to 96-well plates coated with Tkip, DTkip, DRTkip, control peptide (MuIFNγ(95-106)), or buffer alone. The assays were developed using standard ELISA methods with neutravidin-HRP conjugate to detect bound biotinylated JAK2(1001-1013). B) Biotinylated pJAK2(1001-1013) binds to Tkip, DTkip and DRTkip. Biotinylated pJAK2(1001-1013) was added to wells coated with the peptides or buffer and binding assays were carried out as described above. The binding of JAK2(1001-1013) or pJAK2(1001-1013) to Tkip, DTkip or DRTkip, when compared to control peptide was statistically significant as determined by Mann-Whitney signed rank test ($P < 0.02$ and $P < 0.004$, respectively).

A



B

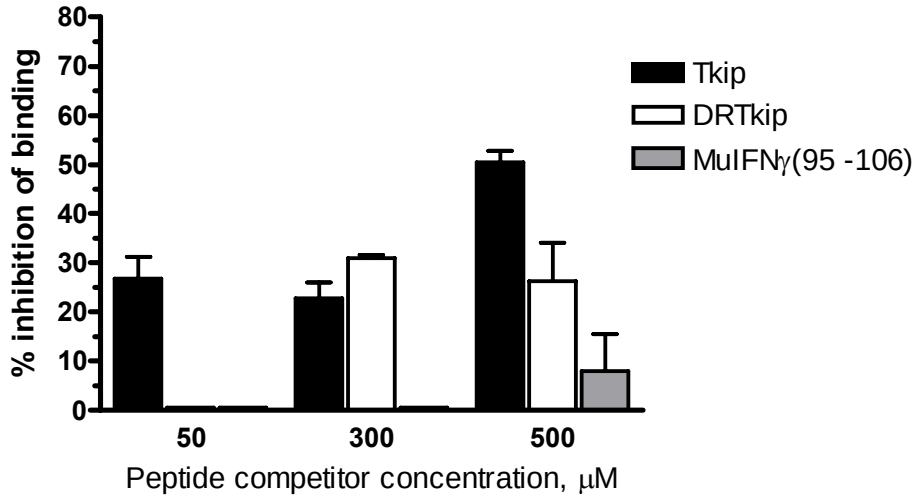


Figure 4-2. Both soluble DRTkip and soluble Tkip inhibit the binding of biotinylated JAK2(1001-1013) and biotinylated pJAK2(1001-1013) to immobilized Tkip. A) DRTkip and Tkip inhibit the binding of JAK2(1001-1013) to immobilized Tkip. The inhibition of binding by Tkip or DRTkip is statistically significant when compared to control peptide (MuIFNγ 95-106) ($P < 0.05$ as determined by Mann-Whitney signed rank test. B) DRTkip and Tkip inhibit the binding of pJAK2(1001-1013) to immobilized Tkip. The differences in inhibition of binding by Tkip to pJAK2, when compared to control peptide, is statistically significant ($P < 0.05$), but that of DRTkip is not statistically significant ($P > 0.05$) as determined by Mann-Whitney signed rank test. All experiments were carried out in triplicate and the data are representative of three independent experiments.

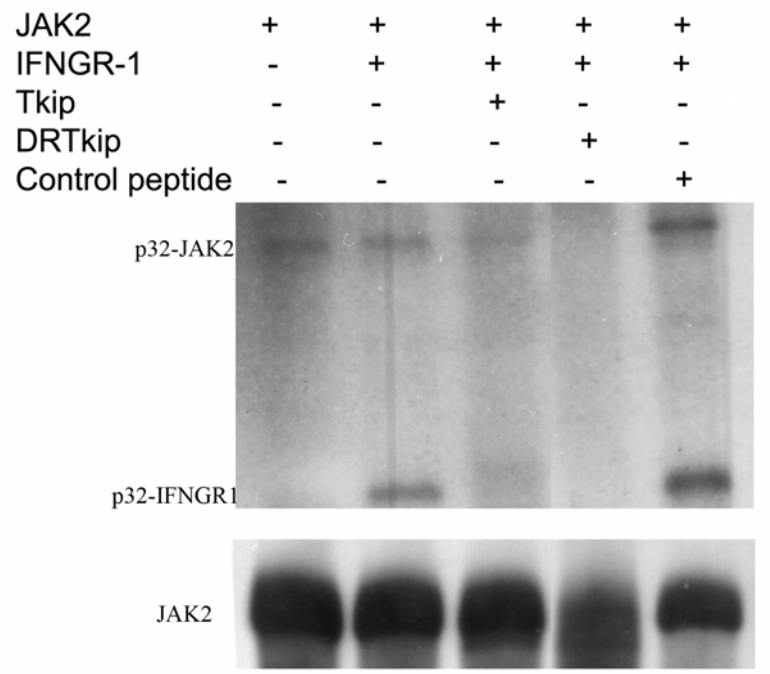


Figure 4-3. DRTkip and Tkip but not the control peptide, MuIFNγ(95-106), inhibit JAK2 autophosphorylation. Kinase assays were carried out in the presence of JAK2 kinase, soluble IFNGR-1, and radiolabelled ATP as described in Chapter 3. To show equal protein loading, an immunoblot with JAK2 antibody of the reactions was carried out as described above, but in the presence of unlabelled ATP.

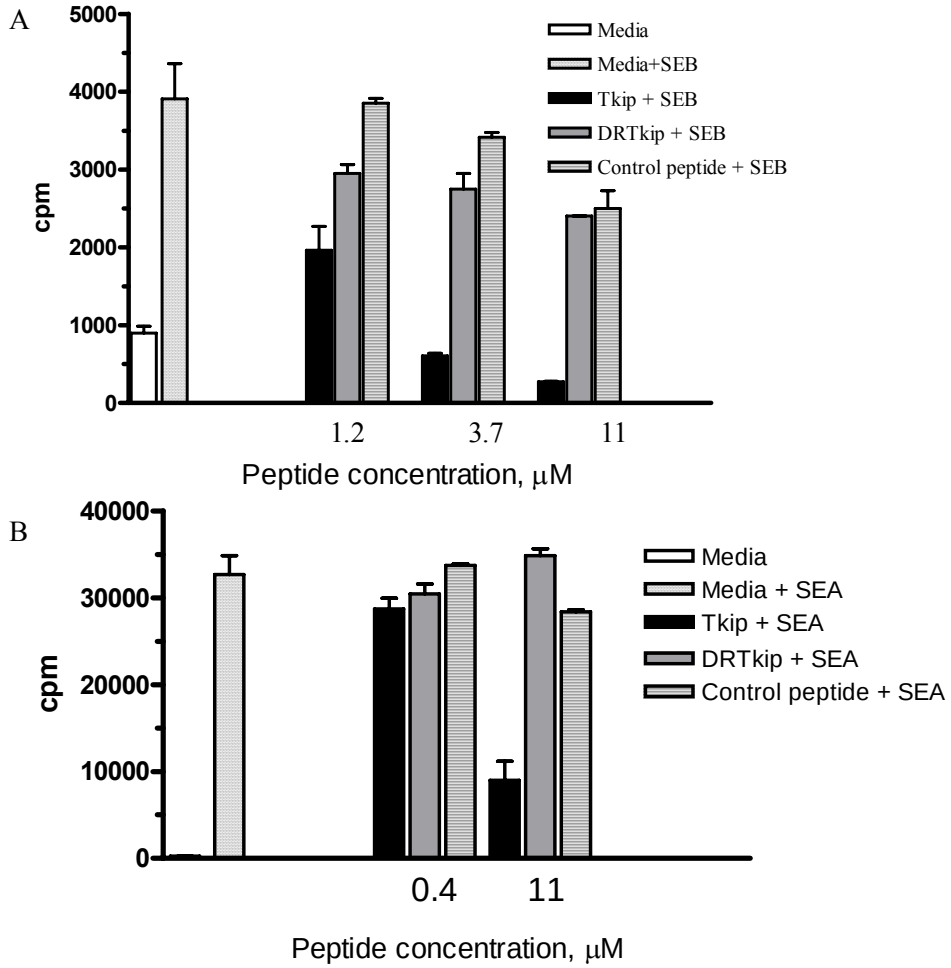


Figure 4-4. Tkip, but not DRTkip inhibits superantigen-induced splenocyte proliferation. A) Tkip, but not DRTkip inhibits SEB-induced splenocyte proliferation. Splenocytes (4 x 10^5 cells/mL) from naïve NZW mice were incubated with varying concentrations of lipophilic peptides Tkip, DRTkip or control peptide MuIFNγ(95-106) and SEB (0.5 μg/mL) for 72 h, followed by pulse labeling with ^{3}-[H]-thymidine, and harvesting on filter discs. Cell associated radioactivity was quantified using a β-scintillation counter and is reported as counts per minute (cpm). Differences between Tkip and control peptide were statistically significant ($P < 0.05$) as determined by Mann-Whitney signed rank test. Differences between DRTkip and control peptide were not statistically significant ($P > 0.05$). B) Tkip, but not DRTkip inhibits SEA-induced splenocyte proliferation. The experiment was carried out as described for SEB, but in the presence of SEA (0.5 μg/mL). The differences between Tkip and control peptide were statistically significant ($P < 0.05$), but the differences between DRTkip and control peptide are not statistically significant ($P > 0.05$) as determined by the Mann-Whitney signed rank test. The experiments were carried out in triplicate and data are representative of three independent experiments.

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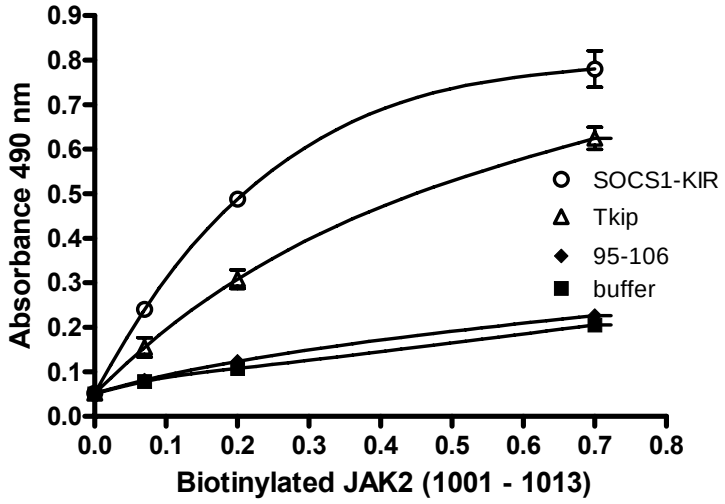

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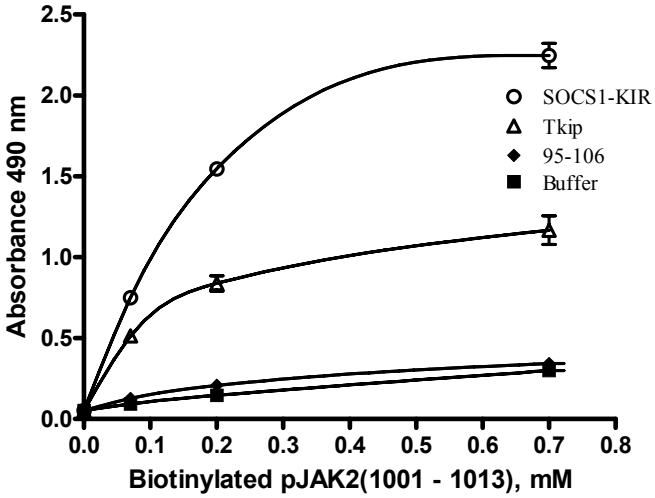

Figure 4-5. JAK2 autophosphorylation site peptides bind to SOCS1-KIR. A) JAK2(1001-1013) peptide binds to both SOCS1-KIR and Tkip. Biotinylated JAK2(1001-1013), at the indicated concentrations, was added in triplicate to a 96-well plate coated with either Tkip, SOCS1-KIR, control peptide (MuIFNγ(95-106)), or binding buffer and binding assays carried out as described in Chapter 3. B) Biotinylated pJAK2(1001-1013) binds to both SOCS1-KIR and Tkip. Biotinylated pJAK2(1001-1013) was added to wells coated with either Tkip, SOCS1-KIR, MuIFNγ(95-106), or buffer and binding assays were carried out as described in Chapter 3. The binding of JAK2(1001-1013) and pJAK2(1001-1013) to Tkip or to SOCS1-KIR, when compared to control peptide were statistically significant ($P < 0.05$) as determined by the Mann-Whitney signed rank test.

Figure 4-6. Both soluble SOCS1-KIR and soluble Tkip inhibit the binding of biotinylated JAK2(1001-1013) and biotinylated pJAK2(1001-1013) to immobilized Tkip or SOCS1-KIR. A) SOCS1-KIR and Tkip inhibit the binding of JAK2(1001-1013) to immobilized Tkip. B) SOCS1-KIR and Tkip inhibit the binding of JAK2(1001-1013) to immobilized SOCS1-KIR. C) SOCS1-KIR and Tkip inhibit the binding of pJAK2(1001-1013) to immobilized Tkip. D**)** SOCS1-KIR and Tkip inhibit the binding of pJAK2(1001-1013) to immobilized SOCS1-KIR. For all competition for binding assays the differences in inhibition of binding by Tkip or SOCS1-KIR, when compared to the control peptide, were statistically significant as Mann-Whitney signed rank test ($P < 0.05$). All experiments were carried out in triplicate and the data are representative of three independent experiments.

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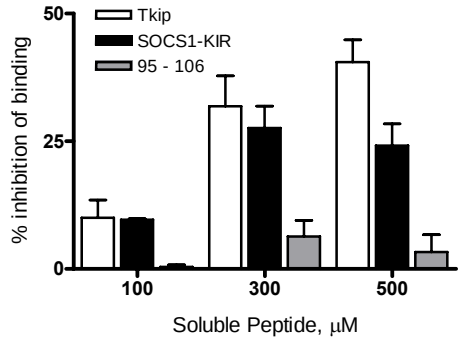
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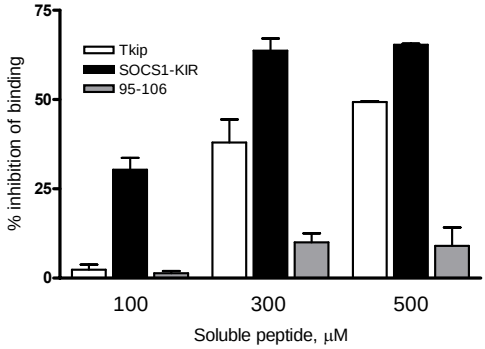
SOCS1-KIR
95 - 106
% inhibition of binding
50
25
0
100
300
500
Soluble Peptide, μM

B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SOCS1-KIR
95-106
% inhibition of binding
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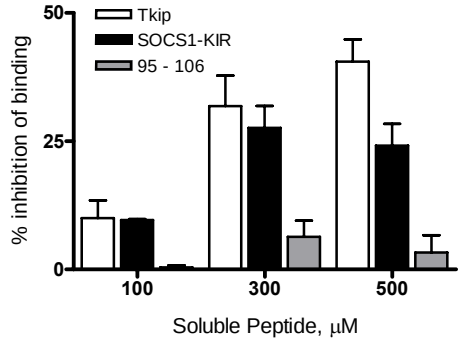
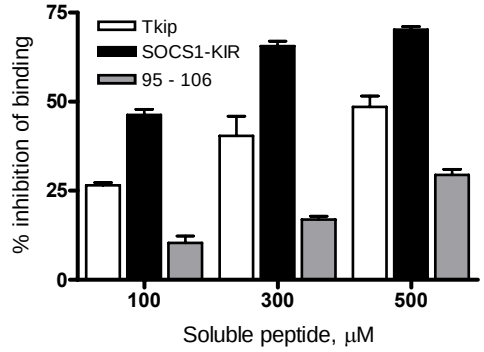
50
25
0
100
300
500
Soluble peptide, μM

C


Tkip
SOCS1-KIR
95 - 106
% inhibition of binding
50
25
0
100
300
500
Soluble Peptide, μM

D


Tkip
SOCS1-KIR
95 - 106
% inhibition of binding
75
50
25
0
100
300
500
Soluble peptide, μM

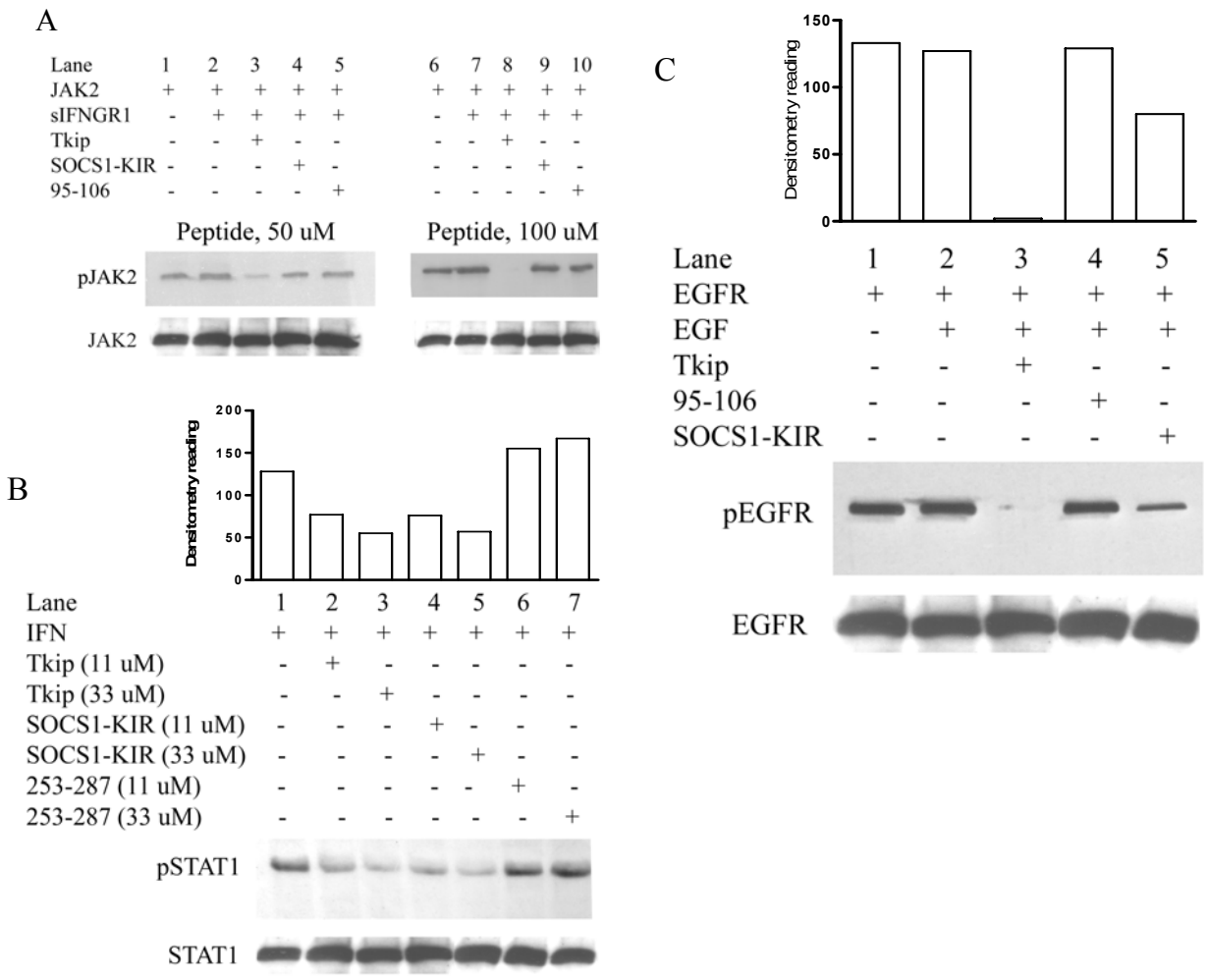
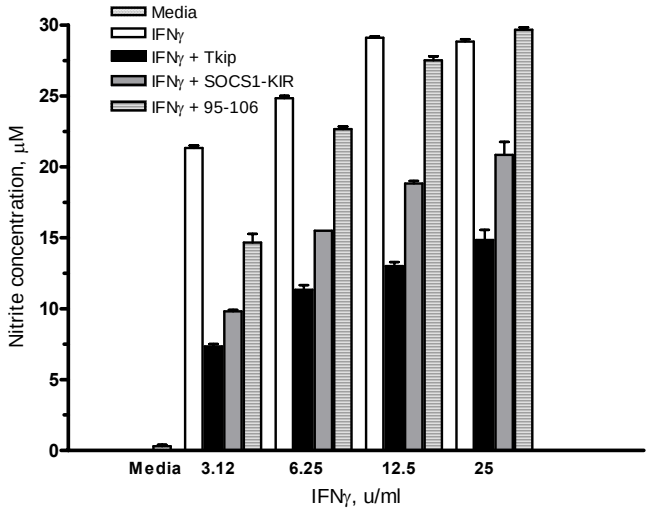


Figure 4-7. Differences in the kinase inhibition patterns of SOCS1-KIR and Tkip in JAK2 autophosphorylation, STAT1α phosphorylation, and EGFR phosphorylation. A) Tkip, but not SOCS1-KIR or the control peptide, MuIFNγ(95-106), inhibits JAK2 autophosphorylation. B) SOCS1-KIR and Tkip, but not the control peptide MuIFGR1(253-287) inhibit IFNγ-induced STAT1α activation in murine U937 cells. Immunoblots with phosphorylated (pY701) STAT1α and the corresponding densitometry readings of band intensities are shown. The membrane was stripped and reprobed with STAT1α antibody. C) Both Tkip and SOCS1-KIR inhibit EGFR phosphorylation. *In vitro* kinase assays were carried out in which SOCS1-KIR, Tkip or control peptide was incubated with EGF and EGFR and ATP for 30 min at 25^0C. The kinase reaction mixtures were resolved on 12% SDS-PAGE, transferred onto a nitrocellulose membrane and immunoblotted with anti-phosphorylated EGFR antibody, with the densitometry readings of band intensities shown. The membrane was stripped and reprobed with EGFR antibody.

A



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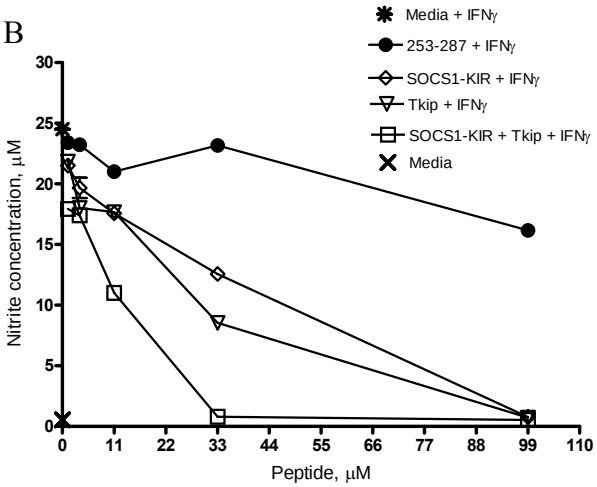


Figure 4-8. SOCS1-KIR and Tkip inhibit IFNγ-induced macrophage activation. A) SOCS1-KIR and Tkip, but not the control peptide significantly inhibited IFNγ-induced macrophage activation as determined by testing for nitrite concentration using Greiss reagent. The inhibition by Tkip, or SOCS1-KIR compared to control peptide was statistically significant as determined by Mann-Whitney rank test ($P < 0.05$). B**)** Tkip, and SOCS1-KIR, but not the control peptide show dose-dependent synergy in inhibiting IFNγ-induced induction of NO. Raw 264.7 cells were treated with IFNγ, in the presence of varying concentrations of Tkip and SOCS1-KIR and screened for NO production as described above. The differences between Tkip or SOCS1-KIR, compared to the control peptide were statistically significant as determined by Wilcoxon matched pairs test ($P < 0.05$).

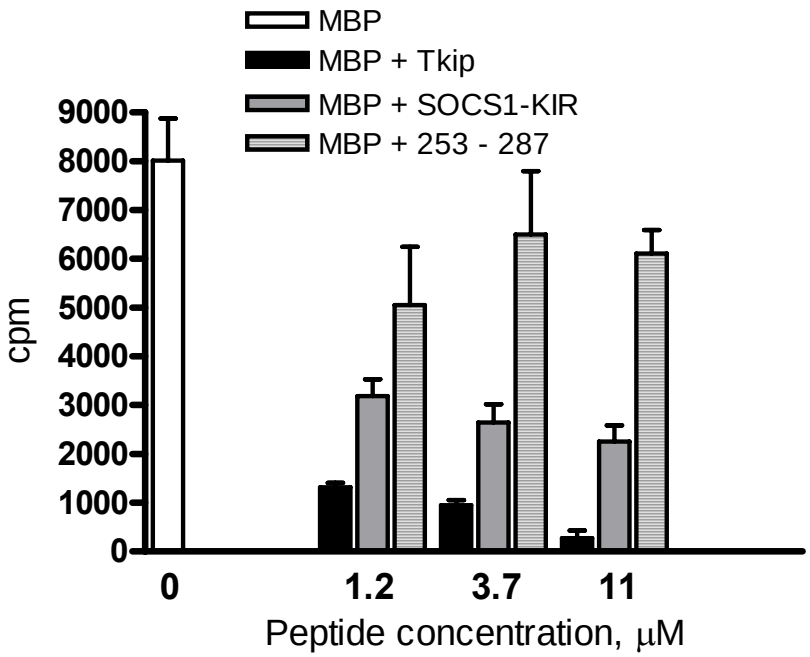


Figure 4-9. Both SOCS1-KIR and Tkip inhibit proliferation of murine splenocytes. Splenocytes (1×10^5 cells/well) were obtained from MBP sensitized SJL/J mice that had developed EAE and were in remission. The splenocytes were incubated with RPMI 1640 complete media containing MBP (50 μg/mL) and varying concentrations of lipophilic SOCS1-KIR, lipophilic Tkip or lipophilic control peptide, MuIFNGR1(253-287), for 48 h. Cultures were then incubated with [^{3}H]-thymidine for 18 h before harvesting. Radioactivity was counted on a β-scintillation counter and data reported as cpm above background (media only). Both lipo-SOCS1-KIR and lipo-Tkip, but not the control peptide inhibited splenocyte proliferation in a dose-dependent manner. The inhibition of proliferation by lipo-SOCS1-KIR or lipo-Tkip, compared to the control peptide, was statistically significant as determined by Mann-Whitney signed rank test ($P < 0.05$). The data are representative of two independent experiments, each carried out in triplicate.

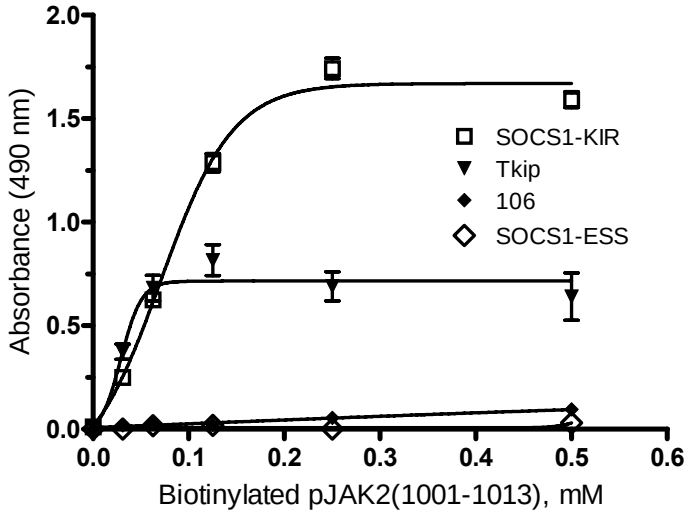
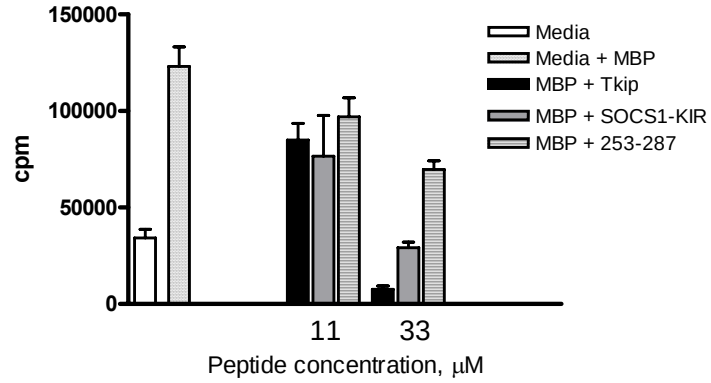


Figure 4-10. Biotinylated pJAK2(1001-1013) binds to SOCS1-KIR but not to SOCS1-ESS. Biotinylated pJAK2(1001-1013) was added to wells coated with either Tkip, SOCS1-KIR, SOCS1-ESS, control peptide MuIFNγ(95-106), or buffer and binding assays were carried out as described in Chapter 3. The binding of pJAK2(1001-1013) to SOCS1-KIR when compared to control peptide was statistically significant as determined by Mann-Whitney signed rank test ($P < 0.01$), while no significant binding was observed between SOCS1-ESS and the control peptide ($P > 0.05$).

A



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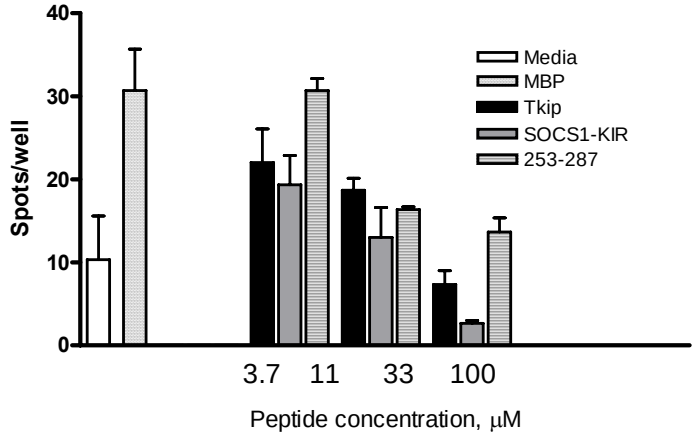

Figure 4-11. Tkip and SOCS1-KIR inhibit antigen-specific $CD4^+$ T cell proliferation and $CD4^+$ T cell-induced IFNγ production. A) Tkip and SOCS1-KIR inhibit $CD4^+$ T cell proliferation. Splenocytes obtained from MBP sensitized SJL/J mice, in remission, were enriched for $CD4^+$ T cells and incubated (5×10^5 cells/well) with varying concentrations of lipophilic peptide in the presence or absence of MBP (50 μg/mL) and APCs for 72 h. The cultures were pulsed with ^{3}[H]-thymidine for 18 h before harvesting. Radioactivity was counted and is reported as cpm. Differences in inhibition of proliferation by Tkip or SOCS1-KIR, compared to control peptide, MuIFNGR1(253-287) were statistically significant ($P < 0.05$) as determined by Mann-Whitney signed rank test. B) Tkip and SOCS1-KIR inhibit $CD4^+$ T cell induced IFNγ production. $CD4^+$ T cells were incubated with lipophilic peptides in the presence or absence of MBP and APCs. The cells were transferred onto IFNγ-ELISPOT plate, and incubated for 48 h. Spots, representing IFNγ-producing cells, were detected using HRP-conjugated secondary antibody. Differences in reduction of the number of IFNγ-producing cells by Tkip or SOCS1-KIR, compared to control peptide, were not statistically significant as determined by Mann-Whitney signed rank test ($P < 0.05$). The experiments were carried out in triplicate and the data are representative of two independent experiments.

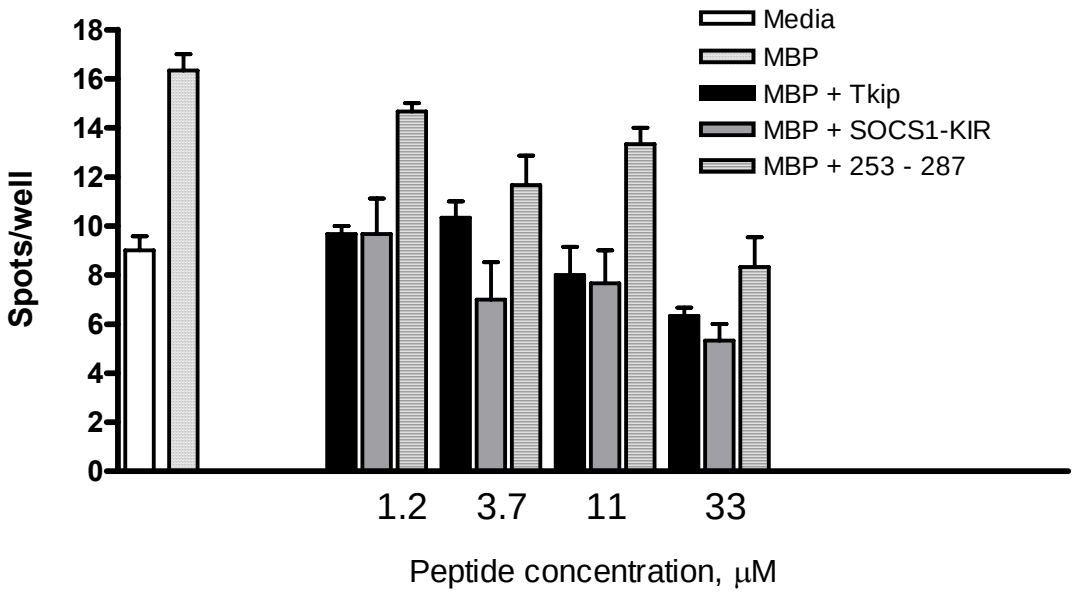

Figure 4-12. Tkip and SOCS1-KIR inhibit $CD8^+$ T cell-induced IFNγ production. Splenocytes were obtained from MBP sensitized SJL/J mice that a few days after signs of active disease. The splenocytes were enriched for $CD8^+$ T cells (5×10^5 cells/well) and incubated with varying concentrations of lipophilic peptides in the presence or absence of MBP (50 μg/mL) and APCs. The cells were transferred into IFNγ-Elispot plates and incubated for 48 h. The spots, representing IFNγ-producing $CD8^+$ T cells, were detected using HRP-conjugated secondary antibody. The differences in inhibition of IFNγ production by Tkip-treated or SOCS1-KIR treated cells, compared to control peptide-treated cells (11 and 33 μM) were statistically significant as determined by Mann-Whitney signed rank test ($P < 0.03$). The experiment was carried out in triplicate and the data are representative of two independent experiments.

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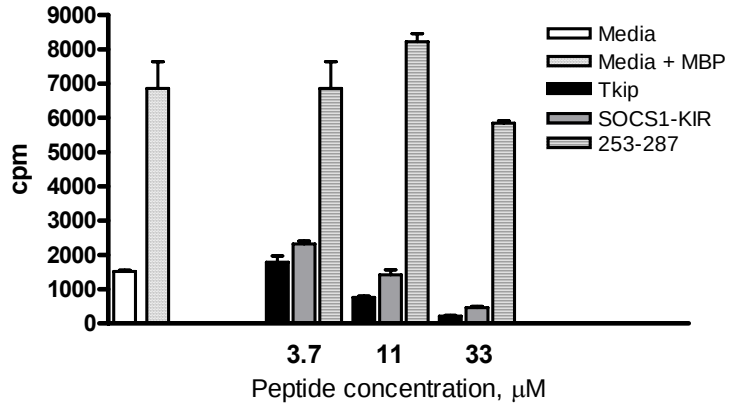

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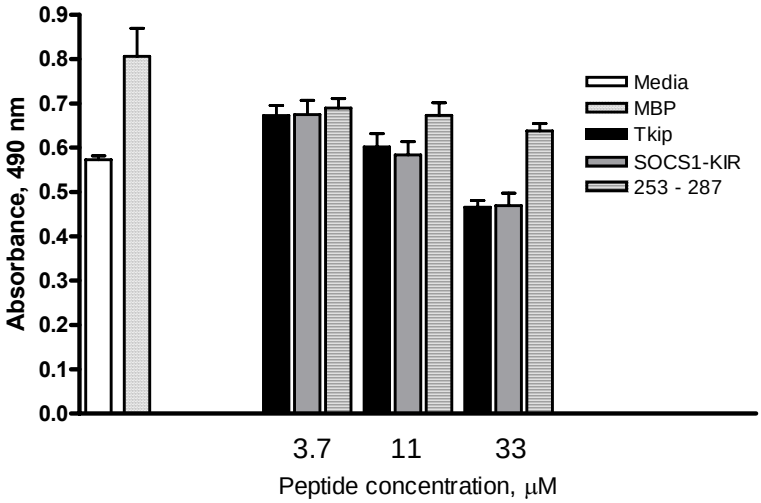


Figure 4-13. Tkip and SOCS1-KIR inhibit antigen-induced B cell proliferation and antibody production. A) Tkip and SOCS1-KIR inhibit B cell proliferation. B cells (5 x 10^5 cells/well) obtained from MBP sensitized SJL/J mice 2 months after last remission were incubated with lipophilic peptides in the presence or absence of MBP and APCs. The data presented for peptides was in the presence of MBP and APCs. The cultures were pulse labeled with ^{3}[H]-thymidine for 18 h and harvested. Radioactivity was counted and is reported as cpm. The differences in inhibition of proliferation by Tkip or SOCS1-KIR (3.7, 11, and 33 μM), compared to the control peptide were statistically significant as determined by Mann-Whitney test ($P < 0.01$). B) Tkip and SOCS1-KIR inhibit the production of MBP-specific antibodies. B cells (5 x 10^5 cells/well) from MBP sensitized SJL/J mice were incubated with lipophilic peptides in the presence of MBP (50 μg/mL) and APCs for 48 h. Culture supernatants were then harvested and tested for MBP-specific antibodies by ELISA. The difference recorded for Tkip or SOCS1-KIR (33 μM), compared to control peptide were statistically significant ($P < 0.01$) as determined by Wilcoxon matched pairs test. The experiments were carried out in triplicate and data is representative of two independent experiments.

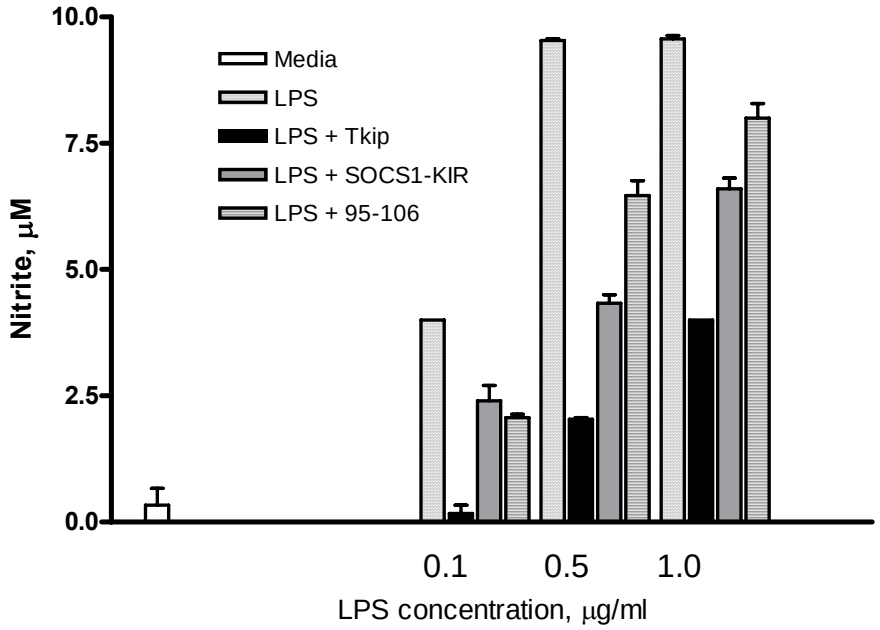
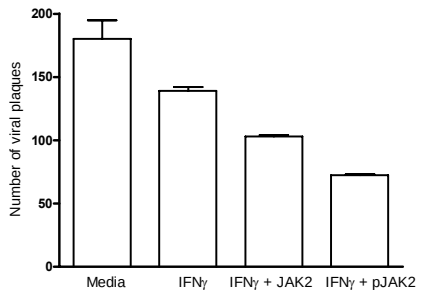


Figure 4-14. Tkip inhibits LPS-induced macrophage activity. Murine macrophage cells, Raw 264.7, were incubated with varying concentrations of LPS alone or with either lipophilic Tkip, lipophilic SOCS1-KIR or lipophilic control peptide, MuIFNγ(95-106), each at 24 μM final concentration, for 48 h. Culture supernatants were collected and nitrite concentration determined using Greiss assay. The experiments were carried out in triplicate and data are representative of two independent experiments. There was statistically significant difference between Tkip and control peptide ($P < 0.05$), but none between SOCS1-KIR and control peptide ($P > 0.05$), at the concentrations tested, as determined by Wilcoxon matched pairs test.

Figure 4-15. pJAK2(1001-1013) peptide has SOCS-1 antagonist properties. A) pJAK2(1001-1013) enhances suboptimal IFNγ-induced antiviral activity against EMC virus in human fibroblast WISH cells as determined using a cytopathic assay. B) pJAK2(1001-1013) reverses SOCS-1 inhibition of STAT3 phosphorylation in human prostate cancer cells (LNCaP) transfected with a SOCS-1 plasmid DNA, pEF-FLAG-I/mSOCS-1, provided by Dr. David Hilton. The results of an immunoblot assay with phosphorylated STAT3 (pY705) and the corresponding densitometry readings of band intensities are shown. Also shown are results of an immunoblot assay with unphosphorylated STAT3 antibody as well as SOCS-1 expression in LNCaP cells. The data are representative of two independent experiments. C) pJAK2(1001-1013) enhances GAS promoter activity. WISH cells were transfected with a vector expressing firefly luciferase, driven by a GAS promoter, along with a vector expressing *Renilla* luciferase as a control vector. IFNγ (1 U/mL) and lipo-pJAK2(1001-1013) at 5 or 1 μM final concentration, or a control peptide, MuIFNγ(95-125) (5 μM) were added to the cells. After 48 h incubation, the cell extracts were assayed for relative luciferase activities using a luminometer. D) Soluble SOCS-1 protein, similar to SOCS1-KIR inhibits the binding of biotinylated pJAK2(1001-1013) to SOCS1-KIR. Biotinylated pJAK2(1001-1013) that had been preincubated with varying concentrations of soluble SOCS1-KIR, SOCS-1 lysate or control lysates, was added in triplicate to a 96 well plate coated with SOCS1-KIR. Bound biotinylated pJAK2(1001-1013) was detected using neutravidin-HRP conjugate as described in Chapter 2. Also shown is an immunoblot lysate showing expression of SOCS-1 in Sf9 insect cells. Aliquots of the cell lysate were used for the competition for binding assay.

A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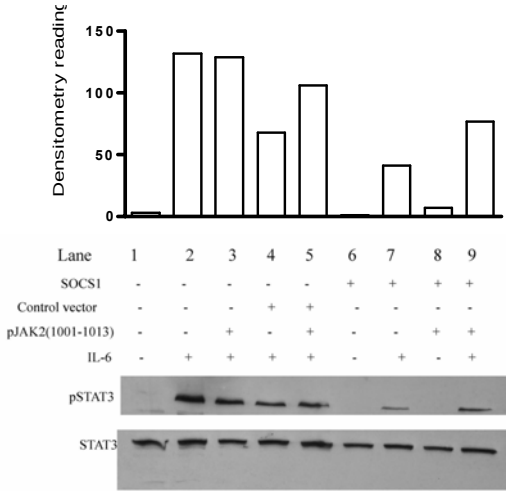
100
50
0
Media
IFNγ
IFNγ + JAK2
IFNγ + pJAK2

B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50
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Lane 1 2 3 4 5 6 7 8 9
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Control vector - - - + + - - - -
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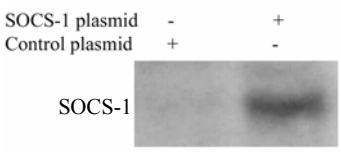
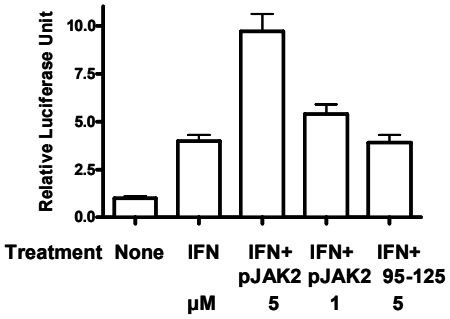
IL-6 - + + + + - + - +
pSTAT3
STAT3


SOCS-1 plasmid - +
Control plasmid + -
SOCS-1

C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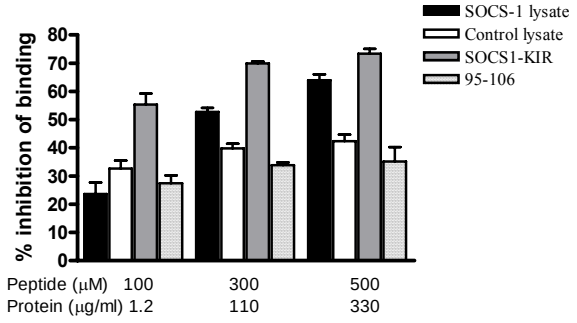
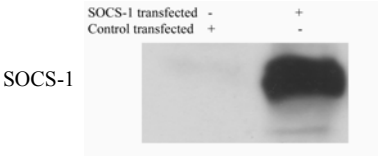
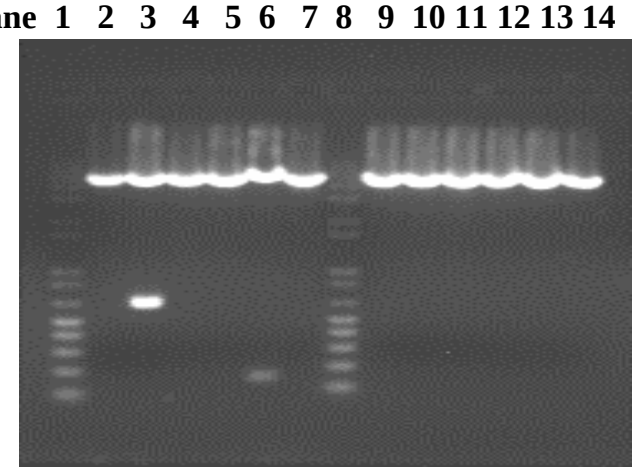
5.0
2.5
0.0
Treatment None IFN IFN+ pJAK2 IFN+ pJAK2 IFN+ 95-125
μM 5 1 5

D


SOCS-1 lysate
Control lysate
SOCS1-KIR
95-106
% inhibition of binding
80
70
60
50
40
30
20
10
0
Peptide (μM) 100 300 500
Protein (μg/ml) 1.2 110 330


SOCS-1 transfected - +
Control transfected + -
SOCS-1

A



B

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   0 MVARNQVAAD NAISPAAEPR RRSEPSSSSS SSSPAAPVRP RPCPAVPAPA
  50 PGDTHFRTFR SHSDYRRITR TSALLDACGF YWGPLSVHGA HERLRAEPVG
 100 TFLVRDSRQR NCFFALSVKM ASGPTSIRVH FQAGRFHLDG SRETFDCLFE
 150 LLEHYVAAPR RMLGAPLRQR RVRPLQELCR QRIVAAVGRE NLARIPLNPV
 200 LRDYLSSFPF QIKGNSKLRP
```

Figure 4-16. SOCS-1 protein was expressed in baculovirus infected Sf9 insect cells. A) The restriction enzyme digestion pattern of pBlueBac/muSOCS1 plasmid DNA with Lane 2 showing the correct restriction enzyme digestion product. B) Amino acid sequence of the cloned plasmid showing full-length SOCS-1 sequence, sequences derived from the cloning vector are underlined.

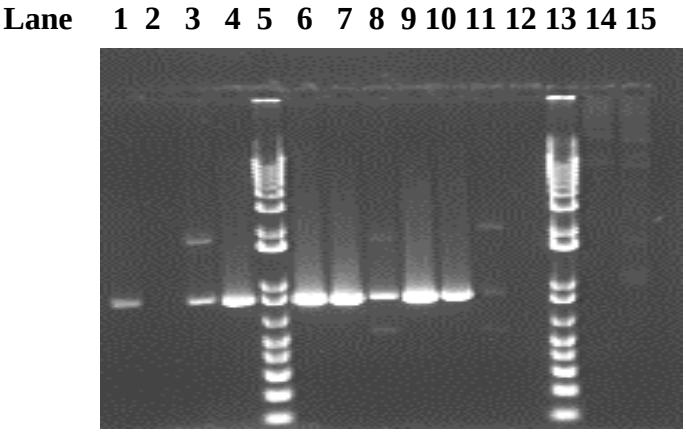


Figure 4-17. Recombinant baculovirus containing muSOCS-1 DNA. PCR amplification of recombinant plaques (blue) with Baculovirus forward and baculovirus reverse primers. Lanes 1, 4, 5, 6, 9, and 10 are carrying inserts of the expected size. Constructs represented by lane 4 and 6 were chosen for further analysis.

CHAPTER 5
DISCUSSION

SOCS-1 is absolutely essential for survival of the individual. Although SOCS-1 knockout mice appear to be normal at birth, they exhibit stunted growth and die neonately by three weeks of age (Alexander et al. 1999). These mice exhibit a syndrome characterized by severe lymphopenia, activation of T lymphocytes, fatty degradation and necrosis of the liver, hematopoetic infiltration of multiple organs, and high levels of constitutive IFNγ as well as abnormal sensitivity to IFNγ (Alexander et al. 1999, Reviewed in Alexander and Hilton 2004, Yoshimura 2005). IFNγ plays a central role in the pathology as SOCS-1 knockout mice that are deficient in IFNγ or IFNγ receptor do not die as neonates. Similar pathology and lethality is observed in normal neonates that are injected with IFNγ. It is worth noting that SOCS-$1^{-/-}$, IFN$\gamma^{-/-}$ double knockout mice die by 6 months of age of severe inflammatory disease (Metcalf et al. 2002) indicating that SOCS-1 regulation is not specific for IFNγ.

The dynamics of induction of SOCS-1 by IFNγ in cells and the activation of STAT1α is illustrative of how SOCS-1 attenuates IFNγ functions under physiological conditions. For example, treatment of monocytes or astrocytes with IFNγ was followed by activation of the SOCS-1 gene at approximately 90 min (Dickensheets et al. 1999, Brysha et al. 2001). Low doses of IFNγ resulted in transient increases in SOCS-1 mRNA that returned to baseline after 4 h, while high concentrations of IFNγ resulted in increases of SOCS-1 mRNA up to 24 h. Thus, the SOCS-1 response appears to be induced by the IFNγ signal. Treatment of hepatocytes from SOCS-$1^{+/+}$ mice with IFNγ resulted in STAT1α activation within 15 min, which peaked by 2 h before declining (Brysha et al. 2001). Although STAT1α is similarly activated in SOCS-1

deficient livers, it persists for 8 h. SOCS-1 thus appears to attenuate IFNγ persistent activation of STAT1α, which nonetheless allows the beneficial effects of IFNγ-induced signaling.

Given the critical importance of SOCS-1 in modulating the activities of IFNγ and other inflammatory cytokines that use tyrosine kinases such as JAK2 in their signaling pathways, our laboratory developed the small tyrosine kinase inhibitor peptide, Tkip, which is a mimetic of SOCS-1 (Flowers et al. 2004). Tkip was designed to recognize the autophosphorylation site on JAK2 involving residues 1001 to 1013 containing the critical tyrosine at 1007 (Yasukawa et al. 1999). Flowers et al (2004) showed that Tkip blocked JAK2 autophosphorylation as well as tyrosine phosphorylation of substrates such as STAT1α and IFNγ receptor chain, IFNGR-1. The authors also showed that like SOCS-1, Tkip blocked EGFR autophosphorylation, while not affecting the tyrosine kinase function of c-Src and vascular endothelial growth factor receptor. Additional experiments showed that Tkip blocked IL-6 induced activation of the STAT3 oncogene in LNCaP prostate cancer cells, which involved inhibition of JAK2 activation (Flowers et al. 2005). These studies presented a proof-of-concept demonstration of a peptide mimetic of SOCS-1 that regulates JAK2 tyrosine kinase function.

Because of its potential for regulation of inflammatory conditions where tyrosine kinases such as JAK2 play a role in the resultant pathology, Tkip was tested in a mouse model of multiple sclerosis called experimental allergic encephalomyelitis (EAE) (Mujtaba et al. 2005). SJL/J mice were immunized with myelin basic protein (MBP) for induction of the relapsing/remitting form of EAE. Tkip, 63 μg every other day, given intraperitoneally, completely protected the mice against relapses when compared to control groups in which greater that 70% of the mice relapsed after primary incidence of disease. Protection of mice correlated with lower MBP antibody titers in Tkip-treated groups as well as suppression of MBP-

induced proliferation of splenocytes taken from EAE-afflicted mice. Consistent with its JAK2 inhibition function, Tkip also inhibited the activity of inflammatory cytokine TNF-α, which uses the STAT1α transcription factor. Thus, Tkip, like SOCS-1, possesses anti-inflammatory activity that protects mice against ongoing relapsing/remitting EAE.

The design of Tkip was independent of any knowledge of the structural and functional domains of SOCS-1. Given that the design focused on Tkip binding to the autophosphorylation site of JAK2, I compared Tkip with regions of SOCS-1 that have been proposed to be either directly involved in such binding or to be involved in enhancement of SOCS-1 binding to the autophosphorylation site. Yasukawa et al. (1999) identified three regions that were directly involved in SOCS-1 binding to JAK2, the large SH2 domain, a N-terminal 12-amino acid sequence called extended SH2 (ESS), and an additional N-terminal 12-amino acid region called the kinase inhibitor region (KIR). The ESS and SH2 domains were felt to bind to the autophosphorylation or activation site of JAK2, while KIR bound to the catalytic site in this model. The 12-amino acid ESS sequence consists of residues 68–79, while KIR consists of residues 56-67. The SOCS1-KIR peptide sequence compared to the KIR above consists of residues 53-68, sharing just the I68 with the ESS and containing three additional residues in its N-terminus. In comparative binding, the SOCS1-KIR peptide bound to pJAK2(1001-1013), while SOCS1-ESS peptide failed to bind (Figure 4-10). Close examination of N-terminal truncated SOCS-1 expressed proteins, designated dN51 (missing residues N-terminal to 51) and dN68, in Yasukawa et al (1999) showed that removal of the KIR region resulted in loss of binding to JAK2 in transfected cells. Further, in direct binding to pJAK2 autophosphorylation site peptide by truncated dN56 SOCS-1 protein, the SOCS1-KIR peptide sequence except for residues 53-55, was present along with ESS and SH2 (Yasukawa et al. 1999). Although the

region of SOCS-1 that binds to the autophosphorylation site of JAK2 as per Yasukawa et al. (1999) is referred to as SOCS-1 SH2 plus ESS, the bindings that they refer to also contained our SOCS1-KIR sequence. Additionally, I have shown that SOCS-1 protein competed with SOCS1-KIR for pJAK2(1001-1013), suggesting that the two recognized the JAK2 autophosphorylation site similarly. Thus, I feel that the binding data with our SOCS1-KIR are consistent with the binding studies of these authors. It should be noted that the peptide binding approach used here does not involve assessment of collaboration and/or synergism among the KIR, ESS, and the SH2 domains of SOCS-1. Thus, based on the studies reported here, along with those by others (Yasukawa et al. 1999), KIR, ESS and SH2 may all be involved in binding to JAK2 autophosphorylation site. It remains to be determined as to the extent of their relative roles.

I have shown in this study that SOCS1-KIR, independent of other domains of SOCS-1, can bind directly to a peptide, JAK2(1001-1013), that corresponds to the autophosphorylation site of JAK2. Further, I showed that SOCS1-KIR competed with Tkip for binding to JAK2(1001-1013). The competitions suggest that the peptides recognized JAK2 similarly but not exactly the same way. Phosphorylation of tyrosine 1007 on the JAK2 peptide enhanced binding of Tkip and SOCS1-KIR. Tkip blocked JAK2 autophosphorylation as well as JAK2 phosphorylation of STAT1α, while SOCS1-KIR did not block autophosphorylation but did block phosphorylation of STAT1α, similar to the pattern or profile of SOCS-1 inhibition of phosphorylation (Alexander and Hilton 2004). The peptides were also functionally similar in inhibiting IFNγ activation of macrophages to produce NO and inhibiting antigen-specific induction of proliferation of splenocytes, with Tkip being the more effective inhibitor. Thus, I have shown here that the KIR region of SOCS-1 can directly bind to the autophosphorylation site of JAK2. These data are consistent with the observation made by Babon et al. (2006) on SOCS-3, which like SOCS-1 has

KIR. These authors showed that the SH2, ESS, and the C-terminal half of KIR directly contacted the phosphotyrosine binding loop of IL-6 receptor, gp130, and that the N-terminal half of KIR likely bound the JAK kinase. Hence, SOCS-1 KIR likely binds directly to JAK2 autophosphorylation site.

Tkip is relatively hydrophobic, while SOCS1-KIR is hydrophilic. However, both peptides have hydropathic profiles that are complementary to that of pJAK2(1001-1013). ESS however, had a hydropathic profile different from Tkip. Tkip was designed to have a hydrophathic profile complementary to that of pJAK2(1001-1013) (Flowers et al. 2004, Weathington and Blalock 2003). Thus, Tkip would recognize primarily hydrophobic residues or groups in the pJAK2 peptide, while SOCS1-KIR would recognize primarily hydrophilic residues or groups. The binding competition could thus be due to a steric interference, which is consistent with differential effects of Tkip and SOCS1-KIR on JAK2 kinase activity.

It has previously been shown that Tkip has potential anti-tumor (Flowers et al. 2005) and anti-inflammatory (Mujtaba et al. 2005) properties. Hence, Tkip may have potential as a therapeutic agent. Here, I have shown that Tkip and SOCS1-KIR directly affect the activity of $CD4^+$ T cells, $CD8^+$T cells, B cells, and macrophages, which provides additional insights of the direct effect that these peptides have on the cells of the immune system. These data further show the probable therapeutic potential of Tkip.

The fact that the KIR region of SOCS-1 can bind directly to pJAK2(1001-1013) raises the possibility that pJAK2(1001-1013) can function as an antagonist of SOCS-1. It has thus been shown here under four different types of experiments that pJAK2(1001-1013) possesses SOCS-1 antagonist properties. First, pJAK2(1001-1013) enhanced suboptimal IFNγ activity. Second, prostate cancer cells transfected for constitutive production of SOCS-1 protein had reduced

activation of STAT3 by IL-6 treatment. pJAK2(1001-1013) reversed the SOCS-1 effect. Third, pJAK2(1001-1013) enhanced IFNγ activation of the luciferase reporter gene via the GAS promoter. Fourth, pJAK2(1001-1013) enhanced antigen-specific splenocyte proliferation. As indicated above, treatment of cells with IFNγ resulted in activation of the SOCS-1 gene in approximately 90 min and it has been proposed that it is associated with the physiological attenuation of the IFNγ response by SOCS-1 (Dickensheets et al. 1999, Brysha et al. 2001) Consistent with this, it has recently been reported that small-interfering RNA inhibition of SOCS-1 expression in bone marrow dendritic cells resulted in enhanced CTL activity and IFNγ production by ELISPOT, culminating in enhancement of anti-tumor immunity (Shen et al. 2004).

I have thus shown here that SOCS1-KIR binds directly to the autophosphorylation site of JAK2, similar to the binding of Tkip SOCS-1 mimetic, which results in inhibition of JAK2 phosphorylation of substrate. This directly identifies a region of SOCS-1 that possesses intrinsic anti-kinase function. Related to this, the autophosphorylation site peptide, pJAK2(1001-1013), functioned as an antagonist of SOCS-1. These findings with SOCS-1 mimetics and antagonists have implications for novel therapeutic approaches to mimicking SOCS-1 for treatment of inflammatory diseases and for suppressing SOCS-1 in order to enhance the immune response against cancer and infectious diseases.

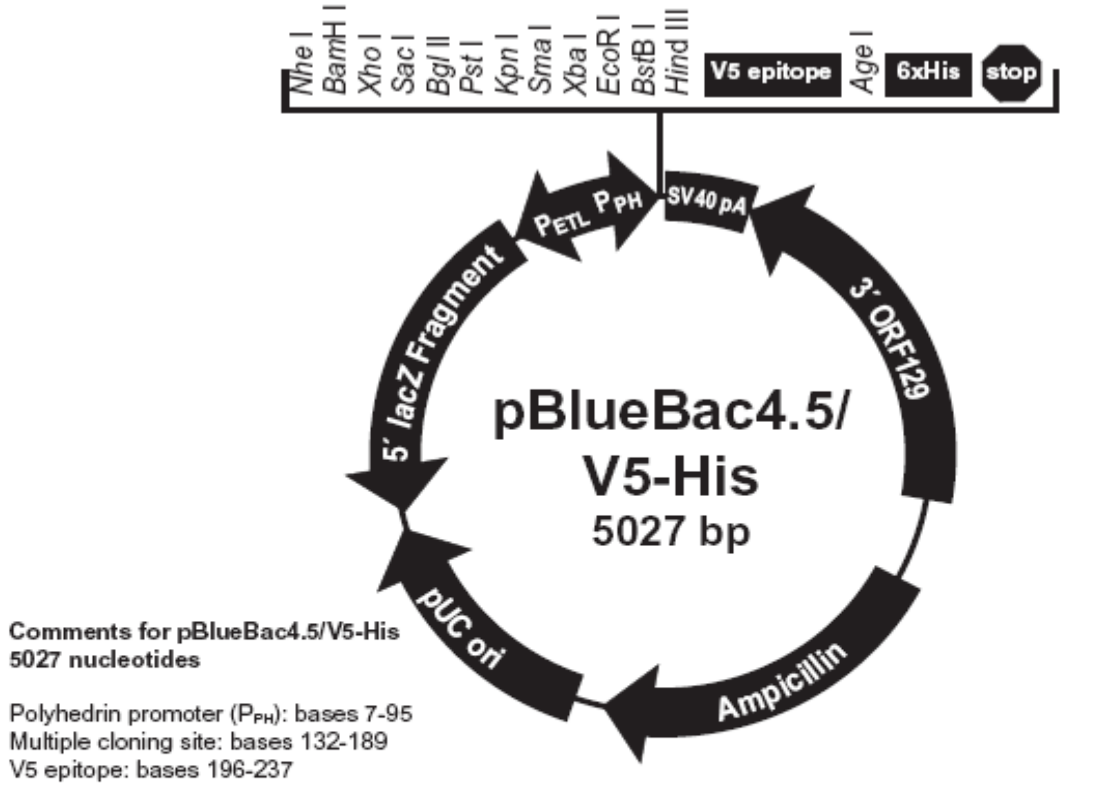
CHAPTER 6
FUTURE WORK

Future work will involve expressing and purifying large quantities of murine SOCS-1 and using the purified protein to characterize the functional regions of SOCS-1 protein. I have also designed additional experiments to determine specifically the role of the SOCS-1 KIR and ESS regions. This would provide additional insight on the minimum domain essential for SOCS-1 activity.

Mouse studies are currently underway attempting to determine whether Tkip can be used for treatment of ongoing relapsing/remitting form of EAE, with implications for treatment of multiple sclerosis. Further, additional experiments are being designed to determine whether Tkip binds to the other JAK kinases, JAK1, JAK3 and TYK2, implication of which Tkip would be used to regulate signaling by these kinases.

For SOCS-1 antagonist studies, mouse studies are being carried out in which attempts are being made to determine whether pJAK2(1001-1013) can enhance protection against ongoing infectious disease.

APPENDIX: VECTOR MAP OF THE TRANSFER (CLONING) VECTOR



Comments for pBlueBac4.5/V5-His
5027 nucleotides

Polyhedrin promoter (P_{PH}): bases 7-95
Multiple cloning site: bases 132-189
V5 epitope: bases 196-237
6xHis tag: bases 247-264
SV40 polyadenylation sequence: 289-416
3′ end of Ac-PH: bases 420-521
3′ end of ORF1629: 1429-551 (complementary)
Sequence homologous to ORF1629 sequence in Bac-N-Blue DNA: bases 633-1431 (complementary)
Ampicillin resistance gene: 1817-2674
pUC origin: 2822-3495
5′ *lacZ* fragment: 4725-3613 (complementary)
Sequence homologous to *lacZ* sequence in Bac-N-Blue DNA: bases 4498-3613 (complementary)
Early to late promoter: bases 5027-4726 (complementary)

Figure A-1. A map of the pBlueBac4.5/V5-His vector. Adapted from pBlueBac4.5/V5-His TOPO Expression Kit manual (Invitrogen).

LIST OF REFERENCES

Ahmed, C. M., M. A. Burkhart, M. G. Mujtaba, P. S. Subramaian, and H. M. Johnson. 2003. The role of IFNγ nuclear localization sequence in intracellular function. *J. Cell Sci. 116: 3089-3098.*

Ahmed, C. M. I., and H. M Johnson 2006. IFNγ and its receptor subunit IFNGR-1 are recruited to the IFNγ-activated genes: Evidence for transactivational activity of IFNGR-1. *J. Immunol. 177: 315-321.*

Alexander, W.S., R. Starr, J. E. Fenner, C. L Scott, E. Handman, N. S. Sprigg, J. E Corbin, A. L Cornish. R. Darwiche, C. M. Owczarek. T. W. Kay, N. A. Nicola, P. J. Hertzog, D. Metcalf, and D. J. Hilton. 1999. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of cytokines. *Cell 98: 597–608.*

Alexander W.S. 2002. Suppressors of cytokine signaling (SOCS) in the immune system. *Nat. Rev. Immunol. 2:410–416.*

Alexander, W.S. and D. J. Hilton 2004. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Ann. Rev. Immunol. 22: 503–529.*

Babon, J. J., E. J. MaManus, S. Yao, D. P. DeSouza, L. A. Mielke, N. S. Sprigg, T. A. Willson, D. J. Hilton, N. A. Nicola, M. Baca, S. E Nicholson, and R. S. Norton. 2006. The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability. *Molecular Cell 22: 205-216.*

Boehn, U., T. Klamp, M. Groot, and J. C. Howar. 1997. Cellular responses to interferon-gamma. *Ann. Rev. Immunol. 15:74 -795.*

Brysha, M., J. G. Zhang, P. Bertolino, J. E. Corbin, W. S. Alexander, N. A. Nicola, D. J. Hilton, and R. Starr. 2001. Suppressor of cytokine signaling-1 attenuates the duration of interferon γ signal transduction *in vitro* and *in vivo. JBC. 276(25): 22086–22089.*

Bromberg, J. and Darnell J.E. Jr. 2000. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene 19: 2468–2473.*

Caló, V., M. Migliavacca M, V. Bazan, M. Macaluso, M. Buscemi, N. Gebbia, and A. Russo A. 2003. STAT Proteins: From normal control of cellular events to tumorigenesis. *J. Cell. Physiol. 197: 157–168.*

Cornish, A. L., M. M. Chong, G. M. Davey, R. Darwiche, N. A. Nicola, D. J. Hilton, T. W. Kay, R. Starr, and W. S. Alexander. 2003. Suppressor of Cytokine Signaling-1 regulates signaling in response to interleukin-2 and other γc-dependent cytokines in peripheral T cells. *JBC 278: 22755-22761.*

Croker, B. A., D. L. Krebs, J. G. Zhang, S. Wormald, T. A. Willson, E. G. Stanley, C. J. Greenhalgh, I. Forster, B. E. Clausen, N. A. Nicola, D. Metcalf, D. J. Hilton, A. W. Roberts, and W. S. Alexander. 2003. *Nat. Immunol. 4: 540-545.*

Darnel, J. E. Jr. 1997. STATs gene regulation. *Science 277:1630–1635.*

Dickensheets, H. L., C. Venkataraman, U. Schindler, and R. P. Donnelly. 1999. Interferon activation of STAT6 by interleukin-4 in human monocytes by induction of SOCS-1 gene expression. *Proc. Natl. Acad. Sci. U.S.A. 96: 10800–10805.*

Durbin, J. E, R. Hackenmiller, M. C. Simon, and D. E. Levy. 1996. Targeted distribution of the mouse STAT1 gene results in compromised innate immunity to viral disease. *Cell 84: 443–450.*

Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misakawa, T. Miyazaki, N. Leonors, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya, and A. Yoshimura. 1997. A new protein containing SH2 domain that inhibits JAK kinases. *Nature 387: 92 –924.*

Feng, J., B. A. Witthuln, T. Matsuda, F. Kohlhuber, I. M. Kerr, and J. N. Ihle. 1997. Activation of JAK2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. *Mol. Cell. Biol. 17(5): 2497 –2501.*

Fenner, J. E., R. Starr, A. L. Cornish, J. G. Zhang, D. Metcalf, R. D. Schreiber, K. Sheehan, D. J. Hilton, W. S. Alexander, and P. J. Hertzog. 2006. Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. *Nat. Immunol. 7: 33-39.*

Flowers, L. O., H. M. Johnson, M. G. Mujtaba, M. R. Ellis, S. M. Haider, and Subramaniam P.S. 2004. Characterization of a peptide inhibitor of Janus kinase 2 that mimics suppressor of cytokine signaling 1 function. *J. Immunol. 172:7510.*

Flowers, L. O., P. S. Subramaniam, and H. M. Johnson. 2005. A SOCS-1 peptide mimetic inhibits both constitutive and IL-6 induced activation of STAT3 in prostate cancer cells. *Oncogene 24(12): 2114–2220.*

Giordanetto, F. and R. T. Kroemer, 2003. A three-dimensional model for the suppressors of cytokine signaling 1 (SOCS1). *Protein Engineering. 16(2): 115–124.*

Hanada, T., T. Kobayashi, S. T. Chinen, T. H. Koga, Y. Minoda, T. Sanada, T. Yoshioka, H. Mimata, S. Kato, and A. Yoshimura. 2006. IFN gamma-dependent, spontaneous development of colorectal carcinomas in SOCS1- deficient mice. *J. Exp. Med. 203(6): 1391-1397.*

Horvath, C. M., G. R. Stark, I. M. Kerr, and J. E. Darnell Jr. 1996. The interactions between STAT and non-STAT proteins in the interferon stimulated gene factor 3 transcription complex. *Mol. Cell. Biol. 19: 5106–5112.*

Ihle, 1995. The Janus protein tyrosine kinases in hematopoietic cytokine signaling. *Semin. Immunol. 7(4): 247–254*.

Imada, K. and W. J. Leonard 2000. The JAK-STAT pathway. *Mol. Immunol. 37: 1-11.*

Jo, D., D. Liu, S. Yao, R. D. Collins, and J. Hawiger 2005. Intracellular protein therapy with SOCS-3 inhibits inflammation and apoptosis. *Nat. Med. 11(8): 892–898.*

Johnson, H. M., P. S. Subramaniam, S. Olsnes, and D. A. Jans 2004. Trafficking signaling pathway of molecular localizing protein ligands and receptors. *Bioessays 26: 993–1004.*

Kile, B.T., N. A. Nicola, and W. S. Alexander 2001. Negative regulators of cytokine signaling. *Int. J. Hematol. 73(3): 29 – 298.*

Kisseleva, T., S. Bhattacharya, J. Schoeder-Braustein, and C. W. Schindler 2002. Signaling through the JAK/STAT pathway. Recent advances for future challenges. *Gene 285:1-24.*

Kotenko, S.V., S. Pestka 2000. JAK-STAT signal transduction pathway through the eyes of cytokine class II receptor complexes. *Oncogene 19(21): 2557–2567.*

Kurzer, J. H., L. S. Argetsinger, Y. J. Zhou, J. K. Kouadia, J. J. O'Shea, and C. Carter-Su 2004. Tyrosine 813 is a site of JAK2 autophosphorylation critical for activation of JAK2 by SH2-Bβ. *Mol. and Cell. Biol. 24(10): 4557-4570.*

Langford, M. P., D. A. Weiget, G. J. Stanton, and S. Baron 1981. Viral plaque-reduction assay for interferon: Microplaque and regular macroplaque reduction assay. *Methods Enzymol. 78: 339–349.*

Larsen, L. and C. Röpke 2002. Suppressors of cytokine signaling: SOCS. *APMIS 110: 833–844.*

Leonard W. J. 2001. Role of JAK kinases and STATs in cytokine signal transduction. *Int. J. Hematol. 73(3): 271–277.*

Li, X., S. Leung, C. Burns, and G. R. Stark 1998. Cooperative binding of STAT1-2 heterodimers and ISGF3 to tandem DNA elements. *Biochem. J. 80: 703-710.*

Lovato, P., C. Brender, J. Agnholt, J. Kelsen, K. Kaltoft, A. Svejgaard, K. W. Eriksen, A. Woetmann, and N. Odum 2003. Constitutive STAT3 activation in intestinal T cells from patients with Crohn's disease. *JBC 278(19): 16777-16781.*

Maier, J., C. Kincaid, A. Pagenstecher, and I. A. Campbell 2002. Regulation of signal transducers and activators of transcription and suppressors of cytokine-signaling gene expression with astrocyte-targeted production of interleukin-12 or experimental autoimmune encephalomyelitis. *American J. Pathol. 160: 271–288.*

Metcalf, D., S. Mitsud, L. Rago, N. A. Nicola, D. J. Hilton, and W. S. Alexander. 2002. Polycystic kidneys and chronic inflammatory lesions are the delayed consequences of loss of the suppressor of cytokine signaling-1 (SOCS-1). 2002. *Proc. Natl. Acad. Sci. U.S.A.. 99(2): 943-948.*

Motulsky, H. Intuitive Biostatics: Oxford University Press, Inc, New York 1995 pp 217-229.

Mujtaba M.G., F. O. Flowers, C. B. Patel, R. Patel, M. I. Haider, and H. M. Johnson 2005. Treatment of mice with suppressor of cytokine signaling-1 mimetic peptide, tyrosine kinase inhibitor peptide, prevents development of acute form of experimental allergic encephalomyelitis and induces stable remission in chronic relapsing/remitting form. *J. Immunol. 175: 5077–5086.*

Naka, T., M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Anono, N. Nashimoto, T. Kajita, T. Taga, K. Yoshizaki, S. Akira, and T. Kashimoto 1997. Structure and function of a new STAT-induced STAT inhibitor. *Nature 387: 924–929.*

Naka, T., T. Matsumoto, M. Narazaki, M. Fujimoto, Y. Morita, Y. Ohsawa, H. Saito, T. Nagasawa, Y. Uchiyama, and T. Kishimoto 1998. Accelerated apoptosis of lymphocytes by augumented induction of Bax in SSI-1 (STAT-Induced STAT Inhibitor-1) deficient mice. *Proc. Natl. Acad. Sci. U.S.A.. 95:15577–15582.*

Parganas, E., D. Wang, D. Stravapodis, D. Topham, J. C. Marine, S. Teglund, E. Vanin, S. Bodner, O. Calamonici, J. M. Deursen, G. Groveld, and J. Ihle. 1998. JAK2 is essential for signaling through a variety of cytokine receptors. *Cell 93: 385–395.*

Rico-Bautista, E., A. Flores-Morales, and L. Fernandez-Perez. 2006. Suppressors of cytokine signaling (SOCS) 2 proten with multiple functions. *Cytokines & Ggrowth Factors Rev. 17: 431-439.*

Shawver, L. K., D. Slamon, and A. Ullrich 2002. Smart drugs: tyrosine kinase inhibitors in cancer therapy. *Cancer Cell 1(2): 117-123.*

Shen, L., K. Evel-Kabler, R. Strude R, and S. Chen 2004. Silencing of SOCS-1 enhances antigen presentation by dendritic cells and antigen specific anti-tumor immunity. *Nature Biotech. 22(12): 1546–1552.*

Shouda, T., T. Yoshida, T. Hanada, T. Wakioka, M. Oishi, K. Miyoshi, S. Komiya, K. I. Kosai, Y. Hanakawa, K. Hoshimoto, K. Nagata, and A. Yoshimura 2001. Induction of the cytokine signal regulator SOCS3/CIS3 as atheurapeutic strategy for treating inflammatory arthritis. *J. Clin. Investig. 108: 178 –1788.*

Shuai, K. 1999. The STAT family of proteins in cytokine signaling. *Prog. Biophy. Molec. Biol. 71:405–1422.*

Shuai, K. 2006. Regulation of cytokine signaling pathways by PIAS proteins. *Cell research 16: 196-202.*

Song, E., S. K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, and J. Lierberman 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med. 9: 347 – 351.*

Starr, R., T. A. Willson, E. M. Viney, L. J. L. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola, and D. J. Hilton. 1997. A family of cytokine-inducible inhibitors of signaling. *Nature 387: 917–921.*

Starr, R., D. Metcalf, A. G. Elefanty, M. Brysha, T. A. Willson, N. A. Nicola, D. J. Hilton, and Alexander W.S. 1998. Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine sinaling-1. *Proc. Natl. Acad. Sci. U.S.A. 95: 14395 – 14399.*

Subramaniam, P. S., B. A. Torres, and H. M. Johnson 2001. So many ligands, so few transcription factors: a new paradigm for signaling through the STAT transcription factors. *Cytokine 15(4): 175–187.*

Suzuki, A. T. Hanada, K. Mitsuyama, T. Yoshida, S. Kamizono, T. Hoshina, M. Kubo, A. Yamashita, M. Okabe, K. Takeda, S. Akira, S. Matsumoto, A. Toyanag, M. Sata, and A. Yoshimura**.** 2001. CIS3/SOC3/SSI3 pays a negative regulatory role in STAT3 activation and intestinal inflammation. *J. Exp. Med. 193: 471–481.*

Szente, B. E., I. J. Weiner, M. J. Jablonsky, N. P. Krishmna, B. A.Torres, and H. J. Johnson 1996. Structural requirement for agonist activity of a murine interferon-γ-peptide. *J. Interferon Cytokine Res. 16: 813–817.*

Tan, J. C. and R. Rabkin. 2005. Suppressors of cytokine signaling in health and disease. *Pediatric Nephrol. 20(5): 567-575.*

Thiam, K., E. Long, C. Verwaerde, C. Auriault, and H. Gras-Masse. 1999. IFNγ-derived lipopeptide: Influence of lipid modification on the conformation and the ability to induce MHC class II expression on murine and human cells. *J. Med. Chem. 42: 3732 –3736.*

Thompson, J. E. 2005. JAK protein kinase inhibitors. *Drug News Persp. 18(5): 305-310.*

Torres, B.A., S. L. Kominsky, G. Q. Perrin, A. C. Hobeika, and H. M. Johnson. 2001. Superantigens: The good, the bad and the ugly. *Exp. Biol. Med. 226: 164–176.*

Ungureanu, D., P. Saharinen, I. Junttila, D. J. Hilton, and O. Silvennoinen. 2002. Regulation of JAK2 through the ubiquitin-proteosome pathway involves phosphorylation of JAK2 on Y1007 and interaction with SOCS1. *Mol. and Cell. Biol. 22(10): 3316-3326.*

Vincentini, C., C. Festuccia, G. L. Gravina, A. Angelucci, A. Marronaro, and M. Bologna 2003. Prostate cancer proliferation is strongly reduced by the epidermal growth factor receptor kinase inhibitor ZD1839 in vitro in human cell lines and primary cultures. *J. Res. Clin. Oncol. 129: 165–174.*

Waiboci, L.W., C. M. Ahmed, M. G. Mujtaba, L. O. Flowers, J. P. Martin, M. I. Haider, and H. M. Johnson. 2007. Both suppressors of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic bind to JAK2 autophosphorylation site: Implications for development of a SOCS-1 antagonist. *J. Immunol. 178: 5058-5068.*

Weathington, N. M., and J. E. Blalock, 2003. Rational design of peptide vaccines for autoimmune disease: harnessing molecular recognition to fix a broken network. *Exp. Rev. Vac. 2: 61-73.*

Yamoaka, K., P. Saharinen, M. Pesu, V. E. Holt III, O. Silvennoinen, and J. J. O'Shea. 2004. The Janus kinaes. *Genome Biology 5: 253–258.*

Yasukawa, H., H. Misawa, H. Sakamoto, M. Masuhara, A. Sasaki, T. Wakioka,S. Ohtsuka, T. Imaizuni, J. N. Ihle, and A.Yoshimura 1999. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding to the activation loop. *EMBO Journal. 18: 1309–1320.*

Yoshimura, A. 2005. Negative regulation of cytokine signaling. *Clin. Rev. All. Immunol. 28: 205–220.*

Yu, .L., D. J. Meyer, G. S. Campbell, A. C. Larner, C. Carter-Su, J. Schwartz, and R. Jove 1995. *Science 269 (5220): 81-83*.

Zhang, J., G. D. Metcalf, S. Rakar, M. Asimakis, C. J. Greenhalgh, T. A. Willson, R. Starr, S. E. Nicholson, W. Carter, W. S. Alexander, D. J. Hilton, and N. A. Nicola. 2001. The SOCS box of Suppressor of Cytokine Signaling-1 is important for inhibition of cytokine action *in vivo*. *Proc. Natl. Acad. Sci. U.S.A. 98(23): 13261-13265.*

BIOGRAPHICAL SKETCH

Lilian Wangechi Waiboci was born on October 5, 1971, in Nyeri, Kenya to Mr. Francis Waiboci Matu and Mrs. Esther K. Waiboci. She grew up in Nyeri, graduating from Bishop Gatimu Ngandu Girls High School in 1989. She earned her B.S. in Biochemistry and Zoology and her M.S. in Biochemistry from the University of Nairobi, Kenya in 1995 and 2001, respectively.

Upon graduating in 1995, Lilian worked as a high school teacher, teaching Chemistry and Biology. During her M.S. program, she was a research assistant at the International Livestock Research Institute (ILRI) and upon completion of her degree requirements worked as a part-time lecturer at Jomo Kenyatta University of Agriculture and Technology (JKUAT), and later for the Walter Reed Army Medical Research Project, HIV Laboratory in Kericho, Kenya as a Laboratory Manager and Research Technician.

Lilian earned her Ph.D. from the Department of Microbiology and Cell Science, University of Florida in May 2007. She will pursue a carrier that includes both aspects of research and teaching in a University or a Research Institute. Lilian is married to Dr. George Muhia Kariuki and they have a son, Victor Kariuki Muhia.