THE THERAPEUTIC EFFECTS OF A SUPPRESSOR OF CYTOKINE SIGNALING 1 MIMETIC PEPTIDE IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS, A MURINE MODEL OF MULTIPLE SCLEROSIS

By

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To my parents, Philip and Patricia Jager
You made me possible.

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Suppressors of cytokine signaling (SOCS) negatively regulate several facets of the immune system by interfering with the Janus kinas/signal transducers and activators of transcription (JAK/STAT) pathway employed by many cytokines. By blocking phosphorylation of the Janus kinase activation loop, the cytokine receptor, and/or the associated STAT, SOCS proteins inhibit downstream intracellular signaling. SOCS also target bound proteins for proteasomal degradation via the SOCS Box, which serves as an E3-ubiquitin ligase. We have developed a small peptide corresponding to the amino acid sequence of the kinase inhibitory region (KIR) of SOCS-1, SOCS1-KIR. This peptide was found to mimic the effects of SOCS-1 *in vitro*.

Using the murine model of relapsing/remitting multiple sclerosis (MS), experimental allergic encephalomyelitis (EAE), we have evaluated the therapeutic effects of SOCS1-KIR *in vivo*. SJL/J mice were given a regimen of SOCS1-KIR or control treatments beginning 12 days post-immunization with myelin basic protein (MBP). While 100% of control mice experienced some form of observable symptoms,

20% of SOCS1-KIR treated mice never became visibly ill. Treated mice that did become sick had significantly lower maximum average disease severity than control mice, and fewer treated mice experienced fatal disease compared to the controls. Additionally, lymphocyte infiltration into the central nervous system (CNS) is reversed and blocked by treatment with SOCS1-KIR.

Interleukin-17 producing T-cells (Th17 cells) have recently been identified as the key effector cells in MS and its murine counterpart EAE. Splenocytes from treated mice were found to produce less IL-17A, both before and after re-stimulation with MBP, and SOCS1-KIR was found to inhibit production of IL-17A by MBP-sensitive splenocytes. Additionally, SOCS1-KIR inhibited MBP-stimulated cell proliferation. SOCS1-KIR was also determined to inhibit production of IL-17A and interferon γ (IFNγ) after stimulation of MBP-sensitized splenocytes with IL-23, a key mediator of Th17 cell function. SOCS1-KIR was shown to inhibit IL-23-induced STAT3 phosphorylation. These results indicate that SOCS1-KIR inhibits cytokine production by Th17 cells and prevents the development of severe relapsing paralysis in mice. Thus, we have developed a peptide that functions as a SOCS-1 mimetic and which has potential therapeutic value with regards to autoimmune diseases like MS.

CHAPTER 1
INTRODUCTION

Suppressors of Cytokine Signaling

Suppressors of cytokine signaling (SOCS) comprise an eight member family of inducible proteins involved in the inhibition of the intracellular signals conveyed when cytokines bind to their cognate receptors on the surface of the cell (Yoshimura et al., 2007). Without regulation, unchecked signaling can lead to autoimmune disorders or cancers. Originally described independently by three separate groups, and thus given a variety of names including STAT-induced STAT inhibitor (SSI) and Janus kinase binding protein (JAB), the SOCS family is currently composed of SOCS-1 through SOCS-7 and the cytokine-inducible SH2 protein (CIS) (Dalpke et al., 2008; Yoshimura et al., 2007; Alexander and Hilton, 2004; Larsen and Ropke, 2002; Inagaki-Ohara et al., 2003). As the names imply, the SOCS proteins are involved in inhibiting a wide array of cytokines, particularly those cytokines that use the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signal transduction pathway (Table 1-1). Also, they are the first known inducible negative feedback inhibitors of type I and type II cytokine receptors, such as those used by interleukins 12 and 23 and the type I and II interferons, respectively (Dalpke et al., 2008; Inagaki-Ohara et al., 2003).

The SOCS Family Proteins

As mentioned above, there are currently eight members of the SOCS family of proteins, including SOCS-1 through SOCS-7 and CIS. SOCS-1 and SOCS-3 are among the most studied members of the family, while the least is known about SOCS-4 (Croker et al., 2008). While there is a great deal of overlap in the SOCS family,

particularly when it comes to what factors are known to induce their expression, it is clear that they each have a unique role to play in the regulation of cytokine signaling.

Expression of SOCS-1 is induced by a wide variety of cytokines, including the type I and II interferons, interleukins such as IL-2, 4, 6, 9, 10, 12 and 21, leukemia inhibitory factor (LIF), granulocyte/macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor-α (TNFα) (Davey et al., 2006; Yoshimura et al., 2007; Croker et al., 2008). Several growth factors and hormones not exclusively tied to the immune system, such as ciliary neurotrophic factor (CTNF), growth hormone, erythropoietin (EPO), prolactin, and insulin, as well as pathogen associated molecules such as lipopolysaccharide (LPS) and CpG DNA can also induce SOCS-1 expression (Davey et al., 2006; Dimitriou et al., 2008).

SOCS-1 is known to be a potent regulator of the type II interferon, interferon γ (IFNγ) (Dalpke et al., 2008; Davey et al., 2006; Yoshimura et al., 2007; Crocker et al., 2008; Baker et al., 2009). In fact, mice deficient in SOCS-1 expression suffer from a lethal autoimmune disease predominantly driven by IFNγ and generally die before weaning (Table 1-2) (Alexander et al., 1999; Davey et al., 2006; Larsen and Ropke, 2002; Yoshimura et al., 2007). SOCS-1 signaling attenuates dendritic cell and macrophage function by decreasing their production of pro-inflammatory cytokines such as TNFα, IFNγ, and IL-12, inhibiting production of nitric oxide in response to toll-like receptor (TLR) stimulation, and decreasing antigen presentation (Yoshimura et al., 2007; Davey et al., 2006; Baker et al., 2009; Dimitriou et al., 2008). SOCS-1 also affects T cell differentiation and activation by inhibiting IFNγ and IL-12 signaling, and

thus blocking T helper 1 (Th1) cells, as well as moderating IL-4 induction of T helper 2 (Th2) cells (Palmer and Restifo, 2009; Dimitriou et al., 2008).

Like SOCS-1, SOCS-2 expression is induced by a wide variety of cytokines, growth factors, and hormones, including the interferons, interleukins 2, 4, 6, and 10, as well as growth hormone, EPO, and insulin (Rico-Bautista et al., 2006; Larsen and Ropke, 2002; Croker et al., 2008). SOCS-2 plays an important role in regulating growth, development, and metabolic activity (Rico-Bautista et al., 2006; Larsen and Ropke, 2002). SOCS-2 is particularly important in the regulation of growth hormone and insulin-like growth factor 1 (IGF-1), and SOCS-2 knockout mice suffer from gigantism, presenting with enlarged organs and bones (Table 1-2) (Rico-Bautista et al., 2006; Croker et al., 2008). However, in contrast to their increased size, SOCS-2 knockout mice are not obese, have decreased bone density compared to wild-type mice, and decreased cortical nerve density (Rico-Bautista et al., 2006).

SOCS-3 expression is induced by most of the same cytokines and growth factors as SOCS-1, and recent work has expanded this list to include interleukins 22, 23, and 27, as well as transforming growth factor β (TGFβ) (Palmer and Restifo, 2009; Tanaka et al., 2008; Croker et al., 2008; Baker et al., 2009; Dimitriou et al., 2008). SOCS-3 is considered to be the main regulator of cytokines that activate JAK/STAT pathways involving the gp130 receptor chain and STAT3, such as IL-6 and LIF, in part because studies have shown that SOCS-3 binds to the gp130 receptor chain and blocks its phosphorylation (Nicholson et al., 2000; Dimitriou et al., 2008; Baker et al., 2009). Because of its role in attenuating IL-6 signaling, SOCS-3 is considered to be a major player in rheumatoid arthritis, which is exacerbated by IL-6, and in the development of T

helper 17 cells, which involves IL-6 and other STAT3 activating cytokines like IL-9 and IL-21 (Dimitriou et al., 2008; Mesquita et al., 2009; Baker et al., 2009, Palmer and Restifo, 2009).

As with SOCS-1, the SOCS-3 knockout phenotype is fatal (Yoshimura et al., 2007; Dimitriou et al., 2008; Inagaki-Ohara et al., 2003; Dalpke et al., 2008; Larsen and Ropke, 2002). SOCS-3 deletion affects the ability of the placenta to form normally, leading to death of the embryo (Fitzgerald et al., 2009; Boyle and Robb, 2008; Dalpke et al., 2008). When conditional knockouts allowing for placental development to proceed normally are used, it can be seen that SOCS-3 has an important role in modulating erythropoeisis in the fetal liver (Dalpke et al., 2008; Larsen and Ropke, 2002). SOCS-3, along with SOCS-1, also functions in the adult liver to regulate insulin sensitivity (Ueki et al., 2004; Boyle and Robb, 2008; Lebrun and Van Obberghen, 2008).

Unlike SOCS-1, 2, and 3, the remaining SOCS proteins, SOCS-4 through 7 and CIS, have not been well studied (Croker et al., 2008; Fitzgerald et al., 2009). There is evidence that SOCS-4 and SOCS-5, which share considerable sequence homology, are both involved in regulating epidermal growth factor (EGF) signaling (Kario et al, 2005; Croker et al., 2008; Palmer and Restifo, 2009). Both SOCS-4 and SOCS-5 expression is induced in fibroblasts upon stimulation with EGF, and they interact with the EGF receptor leading to its degradation (Kario et al., 2005). The precise mechanism of this interaction is not yet known, particularly for SOCS-4, but it may involve the SOCS proteins' SH2 domains (Kario et al., 2005; Croker et al., 2008). SOCS-5 is also known to affect T cell differentiation by blocking IL-4 signaling, thus inhibiting the differentiation of Th2 cells (Palmer and Restifo, 2009). Thus far knockout mice with a SOCS-4

deletion have yet to be developed, but SOCS-5 knockout mice have presented with no observable phenotype (Fitzgerald et al., 2009; Dalpke et al., 2008).

SOCS-6 and SOCS-7, like SOCS-4 and SOCS-5, share a greater sequence homology to each other than to any of the other SOCS proteins (Croker et al., 2008). Both SOCS-6 and SOCS-7 are known to alter signaling by the insulin receptor (Lebrun and Van Obberghen, 2007; Croker et al., 2008; Mooney et al., 2001; Knisz et al., 2009). Although SOCS-6 knockout mice suffer from a slight decrease in growth compared to their wild-type littermates, there does not appear to be any aberration in insulin signaling in these mice (Lebrun and Van Obberghen, 2007; Dalpke et al., 2008; Croker et al., 2008). SOCS-7 knockout mice that do not succumb to hydrocephalus, however, can suffer defects in glucose homeostasis (Fitzgerald et al., 2009; Lebrun and Van Obberghen, 2007; Dalpke et al., 2008). In addition to their role in insulin signaling, SOCS-6 and SOCS-7 have recently been shown to affect T cell receptor (TCR) signaling and mast cell function, respectively (Choi et al., 2010; Knisz et al., 2009; Palmer and Restifo, 2009). SOCS-6 can alter TCR signaling by binding to and targeting the adaptor protein $p56^{lck}$ for proteasomal degradation, thus inhibiting the phosphorylation of the CD3ξ-chain, which is required for transmission of the TCR signal (Choi et al., 2010). SOCS-7, on the other hand, was recently shown to inhibit mast cell degranulation by blocking the signaling cascade initiated by thymic stromal lymphpoietin (TSLP); knocking out SOCS-7 lead to a severe dermatitis associated with increased serum IgE and mast cell infiltration in approximately half of those mice studied (Knisz et al., 2009; Palmer and Restifo, 2009).

CIS expression is induced by a host of cytokines that also up-regulate the expression of SOCS-1 and SOCS-3 (Palmer and Restifo, 2009; Alexander and Hilton, 2004). Yet, while deletion of SOCS-1 or SOCS-3 results in a lethal mutation, the CIS knockout mice have no obvious phenotype (Fitzgerald et al., 2009; Dalpke et al., 2008; Palmer and Restifo, 2009; Larsen and Ropke, 2002). CIS appears to function mainly by modulating JAK/STAT signaling pathways that activate STAT5, such as growth hormone and IL-2 (Ram and Waxman, 2000; Palmer and Restifo, 2009). In fact, transgenic models of mice over-expressing CIS have a similar phenotype to those mice lacking STAT5 (Fitzgerald et al., 2009; Palmer and Restifo, 2009; Dalpke et al., 2008; Alexander and Hilton, 2004; Ram and Waxman, 2000). Mice with these mutations present a phenotype characterized by difficulties in lactation as well as decreased IL-2 signaling and lower numbers of NK and γδT cells (Palmer and Restifo, 2009; Fitzgerald et al., 2009; Dalpke et al., 2008).

Domains of the SOCS Family Proteins and Their Functions

The eight SOCS proteins all share a similar homology in terms of sequence and structure, as shown in Figure 1-1. Beginning with the amino-terminal domains, the eight proteins vary widely in sequence and in length (Yoshimura et al., 2007; Larsen and Ropke, 2002). For example, only SOCS-1 and SOCS-3 have been shown to have a short N-terminal sequence known as the kinase inhibitory region (KIR), although a putative KIR has been described for SOCS-5 by Nicholson's group (Croker et al., 2008). The central and carboxy-terminal domains make up the more conserved regions of the family (Yoshimura et al., 2007; Larsen and Ropke, 2002). The central portion of the proteins is dominated by a SRC homology 2 (SH2) domain, which in the SOCS family has been lengthened N-terminally by a 15 amino acid long extended SH2 (ESS)

domain, a feature not normally found in other proteins that have SH2 domains (Croker et al., 2008). Finally, the carboxy-terminus of the SOCS proteins is composed of a 40-residue sequence designated the SOCS Box.

Each of the above domains has a unique role in the physiological function of the SOCS proteins. The SOCS Box, for example, is known to work in concert with a complex of elongins B and C, cullin-5, RING-box 2, and E2 ubiquitin transferase, to function as an E3 ubiquitin ligase, resulting in the polyubiquitination of the SOCS-bound proteins and their subsequent degradation by the proteasome (Babon et al., 2009, Yoshimura et al., 2007; Croker et al., 2008; Inagaki-Ohara et al., 2003). The SH2 domain and the ESS are involved in directing the SOCS proteins to their respective targets and binding to the phosphorylated tyrosine residues of those proteins. Finally, the KIR sequence is involved in binding to the Janus kinases and inhibiting their ability to phosphorylate their associated receptors and STAT targets (Waiboci et al., 2007; Croker et al., 2008).

SOCS Mediated Inhibition of the JAK/STAT Signaling Pathway

SOCS proteins function as inducible regulators of cytokine signaling by acting as part of a negative feedback loop. In order to appreciate the different mechanisms by which they can inhibit cytokine signaling, it is necessary to first briefly describe the JAK/STAT signal transduction pathway. Janus kinases, like the two-faced god of Roman mythology, are so named because of their ability to respond to what is happening on the outside of the cell, i.e. ligand binding to a receptor, and transmit that information to the inside of the cell, via phosphorylation of their associated proteins. There are four members of the JAK family: JAK1, JAK2, JAK3, and TYK2 (Dalpke et al., 2008; Johnson and Ahmed, 2006). The JAKs are associated in pairs with membrane-

bound cytokine receptors that have no innate tyrosine kinase activity. The current dogma states that when the cytokine binds to the receptor, a conformational shift takes place in the receptor. When this change occurs, the JAKs autophosphorylate their own critical tyrosine residues for activation, and then proceed to phosphorylate their associated receptor, creating a docking site for the signal transducer and activator of transcription (STAT) protein to bind (Johnson and Ahmed, 2006; Dalpke et al., 2008; Larsen and Ropke, 2002). Once the STAT proteins have been recruited to the receptor, they are now in a position to be phosphorylated by the JAKs. The STATs then dimerize to each other and travel to the nucleus where they bind to the DNA and initiate transcription of the cytokine-specific inducible genes, including those of the SOCS proteins responsible for inhibiting the signal (Figure 1-2).

While this is the prevailing doctrine and is widely accepted by the scientific community, it fails to acknowledge that there are only 4 JAKs and 7 STAT proteins. The activated STATs mainly form homodimers for transcriptional function, thus the available combinations are not enough to account for the diversity of signals transmitted by the system, which is involved in the function of over 60 different cytokines, growth factors, and hormones (Subramaniam et al., 2001). Additionally, and STATs lack a nuclear localization sequence (NLS) (Ahmed et al., 2003; Ahmed and Johnson, 2006; Johnson and Ahmed, 2006; Waugh and Hsuan, 2001). Studies addressing this issue have been done on several cytokine signaling pathways, including those of IFNγ, EGF, and CD44 (Fulcher et al, 2008; Subramaniam et al., 2004; Larkin et al., 2000; Lin et al., 2001; Waugh and Hsuan, 2001; Lee et al., 2009). These studies have shown that while the STATs do dimerize upon activation, they do not dissociate from the receptor; rather, a

complex consisting of the cytokine, at least one chain of the receptor, the associated JAK, and the STATs are all translocated to the nucleus, utilizing NLSs that are present in the cytokines, receptors, and/or JAKs.

But no matter how the signal is eventually transmitted to the nucleus, the SOCS proteins have been shown to utilize up to three different mechanisms for inhibiting its transmission (Figure 2-2): (1) The SOCS protein can inhibit the recruitment of STATs to the receptor by binding to the receptor's phospho-tyrosine residues via their SH2 region, thus blocking the JAK-mediated activation of the STATs (Dalpke et al., 2008); (2) the SOCS Box domain present in all of the proteins enables them to target their bound proteins for proteasomal degradation, thus removing them from the signaling system (Babon et al., 2009; Zhang et al., 2001); and (3) the activity of the Janus kinases can also be blocked directly by interaction with the KIR domains found in SOCS-1 and SOCS-3 (Waiboci et al., 2007; Croker et al., 2008). In addition to these well-described mechanisms of action, it has recently been shown that SOCS-1 is present in the nucleus (Baetz et al., 2008; Koelsche et al., 2009; Calabrese et al., 2009; Palmer and Restifo, 2009) and can function as a mediator in the phosphorylation, and thus activation, of the tumor suppressor p53 (Calabrese et al., 2009).

The Loss of SOCS-1 Results in Severe Autoimmune Disease and Death

As can be ascertained from the above discussion, the suppressors of cytokine signaling have an important role in attenuating a variety of different messages cells can receive. Defects in SOCS-mediated regulation of cytokine signaling have been implicated in several cancers and autoimmune diseases (Alexander and Hilton, 2004; Fujimoto et al., 2004; Mujtaba et al., 2005). Therefore, it should come as no surprise that a loss of function of one of these proteins could potentially have devastating

consequences on the individual, as is seen in the cases of SOCS-1, SOCS-2, and SOCS-3 (Table 1-2). Because the work presented herein is focused on the characterization of a peptide that can mimic the function of the SOCS-1 protein, a more complete description of the SOCS-1 knockout mouse is below.

When the SOCS-1 gene has been deleted in mice, the animals succumb early in life to a severe inflammatory disease (Starr et al., 1998; Metcalf et al., 1999; Marine et al., 1999; Alexander et al., 1999; Zhang et al., 2001; Fujimoto et al., 2004; Chong et al., 2005). SOCS-$1^{-/-}$ mice display stunted growth, although they are indistinguishable from their wild-type and heterozygous littermates in the days immediately following birth (Starr et al., 1998; Alexander et al., 1999; Zhang et al., 2001). However, near the end of the first week of life, SOCS-$1^{-/-}$ mice are noticeably smaller than their littermates, and their fur becomes patchy (manuscript in preparation). While the knockouts occasionally do survive until weaning at 21 days, the majority of them have died by 14 days (manuscript in preparation; Starr et al., 1998; Zhang et al., 2001).

Internally, the loss of SOCS-1 leads to monocytic infiltration of several organs, including the liver, pancreas, heart, and lungs (Starr et al., 1998). The thymus is significantly decreased in size, and the number of peripheral T and B cells is severely depleted compared to wild-type pups (Starr et al., 1998; Metcalf et al., 2000). In contrast to their low numbers in the knockout mouse, the activation state of those T cells remaining is high, as indicated by the increased expression of CD25 and CD69 on the surface of $CD4^{+}$ T cells from knockout mice compared to those from their wild-type littermates, and these hyperactive T cells are required for the development of the inflammatory disease seen in the mice (manuscript in preparation; Marine et al., 1999).

Interestingly, there is also an increase in the percentage of regulatory T cells in the thymus, the production of which could potentially be an attempt to stymie the overwhelming effects of the pro-inflammatory T cells (manuscript in preparation).

Interferon γ (IFNγ) is known to be regulated by SOCS-1, and, indeed, it was discovered that SOCS-1$^{-/-}$ mice produce significantly higher levels of IFNγ than their littermates (Alexander et al., 1999; Bullen et al., 2001). Therefore, to study the effects of IFNγ in these mice, SOCS-1$^{-/-}$ mice were treated with anti-IFNγ antibodies and double knockout mice deficient in both SOCS-1 and IFNγ were generated (Alexander et al., 1999; Bullen et al., 2001; Metcalf et al., 2001; Marine et al., 1999). While mice receiving anti-IFNγ antibodies were able to survive longer than untreated knockouts, the monocytic infiltration of multiple organs continued, and the mice eventually died as young adults (Bullen et al., 2001). On the other hand, mice with the double knockout mutation were able to survive much longer, with approximately 50% of the SOCS-1$^{-/-}$ IFNγ$^{-/-}$ mice still alive at 400 days, although it should be noted that this is indicative of a significantly shorter lifespan than wild-type mice (Metcalf et al., 2001). It should be noted, however, that while the double knockout mice avoided the fatty degeneration of the liver seen in the SOCS-1$^{-/-}$ neonates, they were not completely free of disease, with polycystic kidney disease, pneumonia, and chronic skin ulcers beginning as early as 6 months after birth, a syndrome that mimics that seen in murine models of systemic lupus erythamatosus (SLE) (Metcalf et al., 2001; Fujimoto et al., 2004). These experiments indicate that while regulating IFNγ signaling is an important aspect of SOCS-1 function, it is not the only signaling pathway that SOCS-1 modulates.

Multiple Sclerosis and Experimental Allergic Encephalomyelitis

The regulation of cytokine signaling is important in the prevention and control of autoimmune disease. Aberrant cytokine signaling can exacerbate symptoms of autoimmune disorders by encouraging local and systemic pro-inflammatory environments leading to normal tissue destruction (Yoshimura et al., 2007; Schmidt-Weber et al., 2007; Croker et al., 2008; Baker et al., 2009). One example of an autoimmune disease in which normal immune cell signaling and function is disrupted is multiple sclerosis (MS) (Lassmann et al., 2007; Steinman and Zamvil, 2005).

Multiple sclerosis is a demyelinating disease of the central nervous system, in which the axon-insulating myelin produced by oligodendrocytes is degraded by auto-reactive immune cells, producing a loss of neuronal function and eventually leading to the death of the associated neuron (Lassmann et al., 2007; Rodriguez, 2007). MS affects approximately 2.5 million people worldwide, between 250,000 and 400,000 in the United States, and is the leading cause of disability in young Americans between the ages of 20 and 50 (Stuve 2009). Like many autoimmune diseases, women are affected in greater numbers than men, and roughly 2-3 times more women have MS than men.

Experimental Allergic Encephalomyelitis: A Mouse Model of Multiple Sclerosis

The current model used for researching the mechanisms of disease in MS, and for exploring new treatments, is experimental allergic encephalomyelitis (EAE). EAE can be induced in both rats and mice, as well as in non-human primates, and the disease can be initiated by a variety of different methods, such as viral induction with Theiler's murine encephalomyelitis virus (TMEV) or by injecting myelin proteins or peptides

emulsified in adjuvant (Rodriguez 2007; Steinman and Zamvil, 2005; Ercolini and Miler, 2006, Mix et al., 2008; El Behi et al., 2005; Linker and Lee, 2009).

The four clinical courses of MS, relapsing/remitting, primary progressive, secondary progressive, and progressive relapsing, can be mimicked using different methods of inducing EAE; however, none of the existing models of EAE is capable of covering the entire range of disease seen in humans (Herz et al., 2009; Steinman and Zamvil, 2005; Mix et al., 2008; El Behi et al., 2005; Linker and Lee, 2009). While some of the earliest symptoms experienced by patients with MS are difficult to assess in mice, such as fatigue, loss of vision, and sensory disturbances like a “pins and needles” sensation in the limbs, the more overt symptoms such as ataxia and paralysis are readily observable (Lassmann et al., 2007).

Pathological Features of MS and EAE

As discussed above, MS is an inflammatory demyelinating disease, and thus the pathological features of the disease can be divided into those that are neurological and those that are immunological, keeping in mind that the two categories are intimately linked. To begin, the neurological symptoms of MS and EAE are, at the most basic level, due to the loss of neuronal function (Lassmann et al., 2007; Rosenberg et al., 2007; Edgar and Nave, 2009; Herz 2009). This loss of function is initially due to the destruction of the myelin sheath. In the central nervous system, the myelin sheath is a product of oligodendrocytes whose processes encircle the axons of neurons, insulating them and allowing the electrical impulse, known as the action potential, to more quickly travel from the neuron body to the synapse (Herz et al., 2009; Lassmann et al., 2007; Rosenberg et al., 2007; Kandel et al., 2000). Loss of the myelin sheath alters the distribution of sodium channels along the axon in an effort to regain function, which is

initially successful (Herz et al., 2009). However, this leads to a greatly increased demand for adenosine triphosphate (ATP) and a cascade of disregulated and inverted ion gradients across the axonal membrane, eventually resulting in an influx of extracellular calcium that triggers apoptosis of the neuron (Herz et al., 2009; Kandel et al., 2000).

The insulating properties of the sheath are not the only benefit the neurons receive from the oligodendrocytes. Oligodendrocytes, along with other glial cells of the CNS, also secrete nerve growth and survival factors, and regulate nutrient flow into the neurons, helping to maintain white matter homeostasis (Edgar and Nave, 2009; Kandel et al., 2000; Herz et al., 2009). Therefore, oligodendrocytes destruction leads, eventually, to neuronal death.

The immunological features of MS and EAE occur in response to an initiating event, which is currently unknown in MS, that leads to the production and/or activation of auto-reactive, encephalitogenic T cells, which constitute the fundamental source of tissue injury (Lassmann et al., 2007; Rodriguez, 2007; El Behi et al., 2010). In EAE models in which disease is induced by injecting myelin proteins with adjuvant into the mice, the initial response at the site of injection is dominated by dendritic cells and macrophages, which ingest the proteins, process them, and then present them to the adaptive immune cells. Both B and T cells are activated and migrate to the CNS. B cells produce antibodies to the various myelin proteins, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) (Ercolini and Miller, 2006; Antel and Bar-Or, 2003; Rodriguez, 2007; Linker and Lee,

2009). These antibodies opsonize the myelin sheath and promote its destruction by activated macrophages and microglia present at the site of the lesion.

T cells, however, are considered the main perpetrators in the pathology of MS and EAE. For many years, the classical paradigm of T cell mediated pathology suggested that T helper 1 (Th1) cells are the primary effector cells in MS and EAE (Linker and Lee, 2009; Jager et al., 2009; El Behi et al., 2010). Indeed, interferon γ, the cytokine associated with Th1 cells, is required for the breakdown of the blood-brain barrier seen in EAE, and can adversely affect glial cells by inducing apoptosis of oligodendrocytes (Popko et al., 1997; Bettelli and Nicholson, 2000; Stark et al., 2004; Balabanov et al., 2006; Berard et al., 2010; Linker and Lee, 2009; El Behi et al., 2010).

Interferon γ signaling leads to increased permeability of the blood-brain barrier by up-regulating the expression of leukocyte adhesion molecules, such as activated leukocyte adhesion molecule (ALCAM) and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells (Cayrol et al., 2008; Popko et al., 1997; Kebir et al., 2009). Expression of both MHC class I and II is up-regulated on nervous system cells such as astrocytes and oligodendrocytes when treated with interferon γ, increasing the interactions of these cells with immune cells and the probability of death by cell-mediated cytotoxicity (Rodriguez, 2007; Popko et al., 1997; Bettelli and Nicholson, 2000). Macrophages and microglia are both affected by the increased levels of IFNγ seen in MS and EAE, and respond by participating in the damage of the myelin sheath (Hanisch and Kettenmann, 2007; Bettelli and Nicholson, 2000; Rodriguez, 2007). Finally, IFNγ can directly lead to oligodendrocyte death by inducing apoptosis and/or

necrosis, depending on the maturation state of the cell (Balabanov et al., 2006; Popko et al., 1997; Stark et al., 2004).

While these studies show that T helper 1 cells and interferon γ are important in the pathology of MS and EAE, they are not the only immunological processes involved. When mice were treated with anti-IFNγ during EAE, or when IFNγ knockout mice were studied, the animals actually experienced more severe disease than their untreated and wild-type counterparts (El Behi et al., 2010; Bettelli and Nicholson, 2000). Therefore, recent work in MS and EAE has focused on the role of a newly discovered T helper cell subset, the T helper 17 (Th17) cells.

T helper 17 Cells

T helper 17 (Th17) cells are the latest subset of the helper T cell family to be described, which includes T helper 1, T helper 2, and regulatory T cells, to name a few. As with the other subsets, Th17 cells are associated with their own unique cytokine profile, they produce interleukins 17A, 21, and 22, and play important roles in health and disease (Ouyang et al., 2008; McGeachy and Cua, 2008; Mesquita et al., 2009; Korn et al., 2009; Miossec et al., 2009; Awasthi and Kuchroo, 2009). Th17 cells and their cytokines are important in clearing infections of both extracellular and intracellular bacteria, and have been linked to host-protection in systemic *Candida* infections (Ouyang et al., 2008; Awasthi and Kuchroo, 2009). Th17 cell signaling is required for clearance of *Klebsiella pneumoniae* infection of mice, and mycobacteria are known to be potent inducers of a Th17 response (Ouyang et al., 2008; McGeachy and Cua, 2008). On the other hand, Th17 cells have been linked to a number of autoimmune disorders, including multiple sclerosis.

IL-17A is the hallmark cytokine produced by Th17 cells (Korn et al., 2009; Awasthi and Kuchroo, 2009; Miossec et al., 2009; Ouyang et al., 2008; Gaffen, 2008; Xu and Cao, 2010). As can be ascertained from the role of Th17 cells in pathogen clearance, many of the effects of IL-17A signaling are pro-inflammatory in nature. For example, IL-17A stimulates epithelial fibroblast cells to secrete chemokines such as CXCL1 and IL-8, which are potent neutrophil attractants in mice and humans, respectively (Gaffen, 2008; Laurence et al., 2008; Mesquita et al., 2009; Korn et al., 2009; Ouyang et al., 2008; Xu and Cao, 2010). IL-17A also induces the expression several matrix metalloproteases, like MMP9, and adhesion molecules, like intercellular adhesion molecule 1 (ICAM-1), that allow for immune cells to migrate to the site of infection (Ouyang et al., 2008; Gaffen, 2008; Xu and Cao, 2010; Miossec et al., 2009). Additionally, in its protective role, IL-17A stimulates epithelial cells to produce antimicrobial peptides such as β-defensins and mucins, and to produce large amounts of the pro-inflammatory cytokine IL-6, which serves to activate any immune cells that enter the area (Gaffen, 2008; Mesquita et al., 2009; Korn et al., 2009; Ouyang et al., 2008).

IL-17A, however, has also been implicated in the pathology of several autoimmune diseases (Ouyang et al., 2008; Korn et al., 2009; Miossec et al., 2009; Awasthi and Kuchroo, 2009; Xu and Cao, 2010). For example, IL-17A is known to exacerbate rheumatoid arthritis by inducing the aberrant activation of the bone remodeling cells, osteoblasts and osteoclasts, leading to bone erosion and resorption (Miossec et al., 2009). Stimulating the parenchymal cells of the joints to produce MMPs adds to this destruction. IL-17A is also implicated in psoriasis, where keratinocytes respond by

increasing cell proliferation. IL-17A also has a prominent role in multiple sclerosis, as will be described further below. Interestingly, IL-17A appears to have a protective function in autoimmune diseases of the digestive tract, such as Crohn's disease and irritable bowl disease (IBD) (O'Connor et al., 2009; Mesquita et al., 2009).

Currently, IL-17A is thought to signal primarily via a motif with homology to the Toll-IL-1 Receptor-like (TIR) signaling domain, called the SEFIR motif (Shen and Gaffen, 2008; Gaffen, 2008; Ouyang et al., 2008). Much of the work to date has focused on signal transduction downstream of the IL-17A receptor, involving adaptor proteins like Act1 and TRAF6 (Shen and Gaffen, 2008; Moseley et al., 2003; Korn et al., 2009). For example, activation of TRAF6, which is also found in the toll-like receptor (TLR) and IL-1 signaling pathways, leads to the activation of mitogen-activated protein kinase (MAPK) pathways, such as p38 and ERK, and/or the activation of the IKK complex and, subsequently, NF-κB (Shen and Gaffen, 2008; Gaffen, 2008; Moseley et al., 2003; Korn et al., 2009; Ouyang et al., 2008). The transcription factors C/EBPβ and C/EBPδ, which are important for IL-17A induction of IL-6, can be activated via the above adaptor proteins (Shen and Gaffen, 2008; Gaffen, 2008). Additionally C/EBPβ, but not C/EBPδ, can be activated by a signal involving the distal portion of the IL-17A receptor cytoplasmic tail (Shen and Gaffen, 2008; Gaffen, 2008). However, there is evidence that the JAK/STAT pathway is also involved IL-17A signaling, as treatment with IL-17A leads to increased phosphorylation of the Janus kinases, particularly JAK2, and the transcription factor STAT3 in NIH-3T3 fibroblasts (Subramaniam et al., 1999).

In addition to IL-17A, Th17 cells also produce IL-22 (Korn et al., 2009; Awasthi and Kuchroo 2009; Miossec et al., 2009; Aujla and Kolls, 2009; Aujla et al., 2008; Laurence

et al., 2008). A member of the IL-10 cytokine family, IL-22 appears to exert most of its effects at the epithelia of the skin, digestive, and respiratory tissues (Aujla and Kolls, 2009). This tissue tropism is due to the localization of IL-22 receptor expression. IL-22 plays an important role in Th17 cell mediated host defense at the mucosal barrier (Aujla et al., 2008; Laurence et al., 2008; Ouyang et al., 2008). For example, IL-22, via inducing the activation of STAT3, causes epithelial cells to increase the production of endogenous antimicrobial peptides, such as lipocalin-2, and strengthens the integrity of the epithelial layer (Aujla et al., 2008; Laurence et al., 2008; Aujla and Kolls, 2009; Commins et al., 2008). In fact, in a murine model of *Klebsiella pneumoniae* infection, IL-22 not IL-17A was found to be essential for pathogen resistance (Aujla et al., 2008).

Yet, like IL-17A, IL-22 has also been linked to autoimmune diseases. In particular, IL-22 seems to be involved in the pathology of psoriasis, which is not surprising given that keratinocytes are known to express the IL-22 receptor and respond to IL-22 stimulation by proliferating (Aujla and Kolls, 2009; Miossec et al.; 2009; Ouyang et al., 2008). Interestingly, however, it has been shown that IL-22 levels are increased in the cerebrospinal fluid of patients with multiple sclerosis (Kebir et al., 2007; Kebir et al., 2009; Ouyang et al., 2008). It has been suggested that the IL-22 receptor is present on the endothelia of the blood-brain barrier of MS patients, and that IL-22 is in part responsible for the breakdown of the blood-brain barrier in these patients (Kebir et al., 2007), which would be in contrast to the barrier stimulating effects it has in epithelial layers; however, these results were not confirmed in a recent study of the effects of Th17 cells on the blood-brain barrier of mice (Huppert et al., 2010).

Th17 cells also produce IL-21, which signals via the JAK/STAT pathway using a receptor composed of the common IL-2 receptor gamma chain (γ_c) along with the unique IL-21R chain (Sondergaard and Skak, 2009; Korn et al., 2009; Ouyang et al., 2008; Miossec et al., 2009). Like IL-22, IL-21 activates STAT3 phosphorylation, in this case by activating JAK1 and JAK3 (Sondergaard and Skak, 2009; Mehta et al., 2004). However, unlike the IL-22 receptor, which to date has not been found on immune cells (Ouyang et al., 2008; Commins et al., 2008; Aujla and Kolls, 2009), the IL-21 receptor is located almost exclusively on myeloid and lymphoid cells (Sondergaard and Skak, 2009; Ouyang et al., 2008; Mehta et al., 2004; Spolski and Leonard, 2008). Therefore, the main effect of IL-21 stimulation is to modulate the activation state of immune cells in a context dependent manner (Sondergaard and Skak, 2009). For example, B cells have the highest levels of IL-21 receptor expression of any cell type (Sondergaard and Skak, 2009; Ouyang et al., 2008; Spolski and Leonard, 2008). When stimulated with IL-21, B cells that have been co-stimulated with the B cell receptor (BCR) and CD40 will proliferate; however, if B cells have been activated by toll-like receptors (TLRs), IL-21 will induce apoptosis (Sondergaard and Skak, 2009). Similarly, resting mature natural killer (NK) cells have very little IL-21 receptor expressed on their surface, and thus do not respond to IL-21 stimulation; however, when NK cells are treated with IL-2 and/or IL-15, IL-21 stimulation leads to an increase in cytolytic activity and IFNγ production (Sondergaard and Skak, 2009; Mehta et al., 2008). Finally, IL-21 stimulation of naïve $CD4^+$ T cells in the presence of TGFβ leads to the differentiation of these cells in to Th17 cells, whereas IL-21 treatment alone does not (Wei et al., 2007; Sondergaard and Skak, 2009; Spolski and Leonard, 2008; Mehta et al., 2004).

Like IL-17A and IL-22, IL-21 has been considered part of the pathological cytokine profile of many autoimmune diseases (Ouyang et al., 2008; Sondergaard and Skak, 2009; Spolski and Leonard, 2008). For example, one of the defining characteristics of systemic lupus erythamatosus (SLE) is the production of autoantibodies, particularly antinuclear antibodies. As mentioned above, B cells are one of the main responders to IL-21; therefore, it is not surprising that IL-21 should have a role in SLE (Ouyang et al., 2008; Spolski and Leonard, 2008; Sondergaard and Skak, 2009). Indeed, it has been found that polymorphisms in the IL-21 gene are linked to SLE in humans, and, symptoms of SLE in mice can be ameliorated by blocking IL-21 signaling with an anti-IL-21 receptor antibody (Ouyang et al., 2008; Spolski and Leonard, 2008).

Induction, Differentiation, and Maintenance of Th17 Cells

Unlike the Th1 and Th2 subsets, which have relatively straightforward pathways of induction and differentiation from naïve $CD4^+$ T cells, the path followed by the Th17 cells is still being elucidated (McGeachy and Cua, 2008; Awasthi and Kuchroo, 2009; Kaufmann and Kuchroo, 2009; Schmidt-Weber et al., 2007; Louten et al., 2009; McGeachy et al., 2009; Morishima et al., 2009; Chung and Dong, 2009). What is not in dispute is the role of transforming growth factor β (TGFβ). What is under contention is what other cytokines are involved. For example, a combination of TGFβ and interleukin-6 (IL-6) can induce the Th17-specific transcription factor retinoic acid receptor-related orphan receptor-γt (RORγt), but IL-6 is not required to do so (Elyaman et al., 2009; Wei et al., 2007; McGeachy and Cua, 2008; Chung and Dong, 2009). In fact, both IL-9 and IL-21 are capable of replacing IL-6 and working with TGFβ to initiate Th17 cell induction (Elyaman et al., 2009; Wei et al., 2007). Further complicating the

issue, each of these cytokines, IL-6, IL-9, and IL-21, activate STAT3 and induce a positive feedback loop boosting their own production, making it difficult to parse out which cytokine plays what role.

Another cytokine involved in the activity of Th17 cells is interleukin-23 (IL-23) (McGeachy and Cua, 2008; Langrish et al., 2004; Miossec et al., 2009; Korn et al., 2009; Awasthi and Kuchroo, 2009; Kaufmann and Kuchroo, 2009; Schmidt-Weber et al., 2007; Louten et al., 2009; McGeachy et al., 2009; Morishima et al., 2009; Tan et al., 2009; Chung and Dong, 2009; Watford et al., 2004). IL-23 is a member of the IL-12 family of cytokines, and, in fact, it shares the p40 subunit with IL-12 (Langrish et al., 2004; Watford et al., 2004). However, while IL-12 is a dimer of the p40 subunit plus the p35 subunit, IL-23 is a dimer of the p40 subunit plus the p19 subunit. Their respective receptors also share a common subunit, the IL-12Rβ1 chain to which the p40 subunits bind, and have a unique chain, the IL-12Rβ2 and IL-23R chains respectively (Langrish et al., 2004; Watford et al., 2004).

IL-23 is predominantly produced by activated macrophages and dendritic cells, and, like IL-6, IL-9, and IL-21, IL-23 signaling activates STAT3 via the JAK2 and TYK2 kinases, which induces the expression of pro-inflammatory cytokines by macrophages, dendritic cells, natural killer cells, and activated Th17 cells (Langrish et al., 2004; Tan et al., 2009; McGeachy and Cua, 2008; Watford et al., 2004). While IL-23 was determined insufficient to induce the differentiation of naïve $CD4^+$ T cells into Th17 cells, it was recently shown to be required for the terminal differentiation of TGFβ/IL-6 stimulated cells into fully functional Th17 cells (McGeachy et al., 2009). IL-23 receptors are only found on activated effector and memory Th17 cells, not naïve $CD4^+$ T cells, so the naïve

$CD4^+$ T cells must first be primed to express the IL-23 receptor by differentiating cytokines such as TGFβ and IL-6. Therefore, IL-23 signaling is thought to be necessary for stabilizing and maintaining the Th17 phenotype (Awasthi and Kuchroo, 2009, McGeachy et al., 2009; Morishima et al., 2009; Tan et al., 2009; Rowell and Wilson, 2009).

The Role of Th17 Cells in Autoimmunity of the CNS

As discussed above, despite years of research supporting T helper 1 cells as the only and main T helper subset involved in MS and EAE pathology, the discovery of T helper 17 cells has called the dominance of Th1 cells in demyelinating disease into question. Th17 cells have, thus, emerged as the new focus of much of the research being done in EAE.

In patients with MS, cerebrospinal levels of IL-17 and IL-22 were elevated compared to healthy patients, and IL-17 producing T cells have been discovered in lesions in the CNS (Kebir et al., 2007; Ouyang et al., 2008; Aranami and Yamamura, 2008; Louten et al., 2009). To assess the role of IL-17 in EAE, Hofstetter et al. treated mice with anti-IL-17 antibodies (Hofstetter et al., 2005). Mice in the treatment group had significantly reduced severity of disease compared to the control group (Hofstetter et al., 2005). Komiyama et al. studied EAE in IL-$17^{-/-}$ mice (Komiyama et al., 2006). They found that disease progression was delayed and the severity of symptoms was greatly reduced in comparison to wild-type mice (Komiyama et al., 2006).

In light of its important role in Th17 cell differentiation and function, the role of IL-23 in EAE has also been studied. McGeachy et al. showed that IL-23 signaling is necessary for the development of EAE, as mice lacking a functional IL-23 receptor were completely protected from disease (McGeachy et al., 2009). This study, along with that

by Gyulveszi et al. indicates that IL-23 signaling is important in the accumulation of lymphocytes in the CNS, a hallmark of both MS and EAE (McGeachy et al., 2009; Gyulveszi et al., 2009).

Finally, a subset of Th17 cells that express IFNγ has recently been discovered (Abromson-Leeman et al., 2009; Kebir et al., 2009). These cells are positive for both T-bet, the traditional Th1 cell transcription factor, and RORγt, the Th17 specific transcription factor, and, in culture, they can behave as Th1 cells, Th17 cells, or a combination of the two (Abromson-Leeman et al., 2009). IFNγ has been described as inhibiting Th17 cell induction and differentiation, similar to the way in which Th1 cell signaling inhibits the induction of Th2 cells, and most protocols for inducing the Th17 cell phenotype from naïve $CD4^+$ T cells require the addition of neutralizing antibodies for both IFNγ and IL-4 (McGeachy and Cua, 2008; Miossec et al., 2009; Rowell and Wilson, 2009). Yet $IFN\gamma^+$ Th17 cells have been found in patients with MS, and they preferentially accumulate in the CNS (Kebir et al., 2009). Interestingly, these cells cross the blood-brain barrier using ICAM-1, which is primarily up-regulated on brain epithelial cells by exposure to IFNγ, not IL-17 (Kebir et al., 2009; Cayrol et al., 2008). These studies show that more work needs to be done to definitively elucidate the contributions of Th1, Th17, and Th1/Th17 cells and their cytokines in the development and progression of MS and EAE.

Current Treatment Options for Multiple Sclerosis

There is no cure for multiple sclerosis, although the course of disease and symptoms can be managed with a variety of different therapies (Javed and Reder, 2006; DeAngelis and Lublin, 2008; Stuve, 2009). Currently, 4 out of the 5 first-line disease modifying agents prescribed to MS patients are preparations of interferon β, the

fifth being glatiramir acetate (Javed and Reder, 2006). While it may seem anti-intuitive to treat an autoimmune disease with an otherwise pro-inflammatory cytokine, IFNβ also has immunomodulatory effects. IFNβ can inhibit MHC class II expression by antigen presenting cells, decrease T cell activation by inhibiting the production of IL-2, and inhibit the release of IFNγ, which is known to play a role in the pathology of MS (Javed and Reder, 2006). Additionally, IFNβ can inhibit the breakdown of the blood-brain barrier and down-regulate expression of adhesion proteins and matrix metalloproteinases necessary for T cell trafficking into the CNS (DeAngelis and Lublin, 2008; Kraus et al., 2004; Veldhuis et al., 2003). Interferon treatment is not, however, without risk (Borg and Isenberg, 2007; Stuve, 2009). Patients on long-term interferon therapy are at risk for developing injection site reactions, flu-like symptoms, thyroid disease and systemic lupus erythamatosus (SLE) (Borg and Isenberg, 2007; Stuve, 2009).

Other therapies have tried to block specific cytokine receptors or adhesion molecules. For example, natalizumab is a monoclonal antibody to the α4β1 integrin involved in leukocyte migration across the blood-brain barrier (Ransohoff, 2007; DeAngelis and Lublin, 2008; Steinman and Zamvil, 2005). Unfortunately, in addition to blocking the transmigration of encephalitogenic T cells, in some patients natalizumab also blocked entry into the CNS by T cells that were controlling latent JC virus infections, resulting in the development of progressive multifocal leukoencephalopathy and eventual death (Ransohoff, 2007; Steinman and Zamvil, 2005).

Treatment with alemtuzumab, a monoclonal antibody to CD52, which is found on the surface of T cells, natural killer cells, and B cells, leads to a profound and sustained

depletion of T cells (Hauser, 2007; DeAngelis and Lublin, 2008). Patients receiving alemtuzumab experienced decreases in relapse and disability, as well as improved markers of inflammation as viewed by MRI; however, long-term effects of treatment include the development of autoimmune thyroiditis and idiopathic thrombocytopenia purpura (Hauser, 2007; DeAngelis and Lublin, 2008).

A clinical trial using ustekinumab, a monoclonal antibody against the p40 subunit shared by both IL-23 and IL-12, was recently undertaken (Segal et al., 2008; Martin, 2008; Longbrake and Racke, 2009; Steinman, 2010). In this study, ustekinumab showed no significant improvement in the number of CNS lesions viewed by MRI over the course of the trial, nor did the patients report improvement in relapses or disability (Segal et al., 2008; Longbrake and Racke, 2009). This trial and the results of the other drug studies mentioned above highlight the difficulties in treating an autoimmune disease such as multiple sclerosis. The drugs cannot go so far as to destroy the body's ability to defend itself. The treatments cannot perturb the immune system in such a way that it begins to attack another aspect of self. Finally, not all good ideas from the animal models will have success in human patients. Therefore, it is important to continue researching new therapeutic approaches.

Rationale and Specific Aims

Our laboratory has shown that small peptides can mimic the activity of SOCS-1 *in vitro* and *in vivo* (Flowers et al., 2004; Mujtaba et al., 2005; Waiboci et al., 2007; Frey et al., 2009; Ahmed et al., 2009). The original SOCS-1 mimetic, tyrosine kinase inhibitory peptide (Tkip), was designed based on its hydropathic complementarity to the autophosphorylation site of JAK2 (Flowers et al., 2004). Tkip has been shown in vitro to bind to JAK2, to block the phosphorylation of JAK2, and to block phosphorylation of the

IFNγ receptor subunit IFNGR-1 (Flowers et al., 2004). Tkip is also capable of inhibiting the phosphorylation of STAT1 in response to IFNγ, and the phosphorylation of STATs 1, 2 and 3 by the type I interferon IFNτ (Flowers et al., 2004; Ahmed et al., 2009).

Besides the work done in cell culture, Tkip has also been tested in animal models of poxvirus infection and multiple sclerosis (Ahmed et al., 2009; Mujtaba et al., 2005). In response to a lethal dose of vaccinia virus, mice treated with 50 to 200 µg Tkip intraperitoneally prior to infection had an 80-100% survival rate (Ahmed et al., 2009). Treatment with Tkip is also protective against acute and relapsing/remitting EAE (Mujtaba et al., 2005). When Tkip (63 µg/mouse) was administered every other day beginning on the day of EAE-induction, both the incidence of disease and the severity of the symptoms were inhibited compared to the control groups (Mujtaba et al., 2005).

However, in spite of its successes both *in vitro* and *in vivo*, Tkip is a difficult peptide with which to work. In particular, Tkip is difficult to solubilize and easily forms aggregates that precipitate in cell culture media, hindering the ability to consistently reproduce results. Additionally, these solubility issues are problematic when it comes to using Tkip as a therapeutic treatment, because when it forms aggregates in the injection vehicle PBS, it is difficult to precisely determine the amount of peptide being given to the animals. Aggregation and subsequent precipitation, in addition to degradation by serum and tissue protease, are major obstacles to bringing protein and peptide therapeutics to clinical trial (Frokjaer and Otzen, 2005; McGregor, 2008; Sato et al., 2006). Therefore, in addition to Tkip, our laboratory has developed another peptide that can mimic the function of SOCS-1. This peptide, SOCS1-KIR, is based on the sequence of the SOCS-1 kinase inhibitory region (KIR) (Waiboci et al., 2007; Ahmed et

al., 2009). SOCS1-KIR is less hydrophobic than Tkip and is less apt to form aggregates in culture or in PBS (Waiboci et al., 2007). Like Tkip, SOCS1-KIR has been shown to impart a similar level of protection against murine poxvirus infection (Ahmed et al., 2009). It has not, however, yet been tested with regards to EAE.

We hypothesize that the peptide SOCS1-KIR can function in a similar manner to Tkip, and therefore mimic SOCS-1 activity, both *in vitro* and *in vivo* in EAE. The first aim of this study involves examining the ability of SOCS1-KIR to bind to the Janus kinases JAK2 and TYK2, specifically via binding to their autophosphorylation sites. We will then determine the ability of SOCS1-KIR to inhibit Janus kinase activity by analyzing interferon induced STAT phosphorylation. The effects of SOCS1-KIR on antigen-induced cell proliferation using the superantigen *Staphylococcal* enterotoxin B, as well as cytotoxicity, will be addressed. Finally, we will assess the *in vivo* activity of SOCS1-KIR by analyzing its ability to rescue the lethal SOCS-$1^{-/-}$ mice.

The next aim of this study is to describe the therapeutic efficacy of SOCS1-KIR in EAE. To achieve this goal, SJL/J mice will be immunized with myelin basic protein to induce EAE. Treatment with SOCS1-KIR will begin twelve days post-immunization, after lymphocyte infiltration of the CNS but before observable symptoms begin. We will monitor symptoms daily to determine the ability of peptide treatment to alter the course of disease. We will also look at the expression of SOCS-1 mRNA in $CD4^+$ T cells and non-$CD4^+$ monocytes that have infiltrated the CNS. Finally, we will determine the ability of SOCS1-KIR to inhibit MBP-specific cell proliferation using splenocytes isolated from mice with active EAE.

As discussed above, Th17 cells have recently usurped Th1 cells as the purported promoters of pathogenesis in MS as well as EAE (Ouyang et al., 2008; Korn et al., 2009; Miossec et al., 2009; Awasthi and Kuchroo, 2009). Therefore, the final aim of this study will address the effect of SOCS1-KIR treatment on Th17 cell activity in EAE. First, we will determine if the ability of SOCS1-KIR treatment to inhibit the production of the Th17 cell effector cytokine IL-17A by splenocytes isolated from SOCS1-KIR treated mice. Then, we will determine if splenocytes from those mice are able to respond as strongly to the presence of antigen as cells taken from control mice with active EAE. We will also address the ability of SOCS1-KIR to inhibit the production of IL-17A in response to antigen presentation. Because it has been shown that IL-23 is essential for the Th17 cell-mediated pathogenesis of EAE (McGeachy et al., 2009) we will also determine the ability of SOCS1-KIR to inhibit the production of IL-17A and IFNγ in response to IL-23 stimulation. Finally, in order to determine the ability of SOCS1-KIR to directly inhibit the signaling pathways of IL-17A and IL-23, we will analyze the phosphorylation of STAT3 in splenocytes from mice with active EAE after treatment with these cytokines.

Table 1-1. MS/EAE-associated cytokines inhibited by the SOCS family of proteins, with their associated JAKs and STATs.

Cytokines	JAK	STAT
IFNα/β	JAK1, TYK2	STAT1, 2, 3
IFNγ	JAK1, JAK2	STAT1
IL-2	JAK1, JAK3	STAT1, 3, 5
IL-6	JAK1	STAT3
IL-9	JAK1, JAK3	STAT3, 5
IL-12	JAK2, TYK2	STAT3, 4
IL-10	JAK1, TYK2	STAT1, 3, 5
IL-17	JAK1, JAK2, JAK3	STAT 1, 2, 3
IL-23	JAK2, TYK2	STAT3, 4

Table 1-2. Phenotypes of SOCS knockout mice.

SOCS	Phenotype
SOCS-1	Neonatal lethality, lymphopenia, multi-organ monocytic infiltration, fatty liver necrosis, increased IFNγ production and sensitivity
SOCS-2	Gigantism, decreased bone and neuronal density
SOCS-3	Embryonic lethal, placental insufficiency, abnormal erythropoeisis
SOCS-4	Knockout mice have not yet been reported
SOCS-5	No observable phenotype
SOCS-6	Slight decrease in growth
SOCS-7	Increased rates of hydrocephalus, hypoglycemia, dermatitis due to activated mast cells
CIS	No observable phenotype

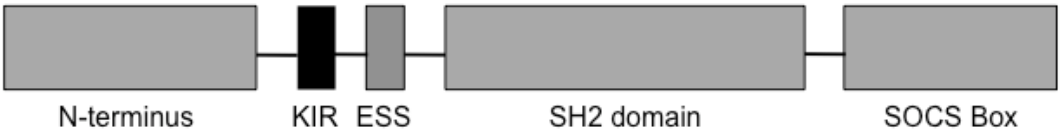

Figure 1-1. Domains of the SOCS-1 protein. All SOCS proteins contain an N-terminus of variable length, an SH2 domain for binding to targeted phosphotyrosine-containing proteins that has additional N-terminal residues over the norm, labeled the extended SH2 domain (ESS), and a SOCS Box domain that is involved in the polyubiquitination of the target proteins, leading to their destruction by the proteasome. SOCS-1, as shown above, as well as SOCS-3 and potentially SOCS-5, has another functional domain known as the kinase inhibitory region (KIR) that interacts with the Janus kinase activation loop, inhibiting the JAK's ability to phosphorylate its target STAT protein.

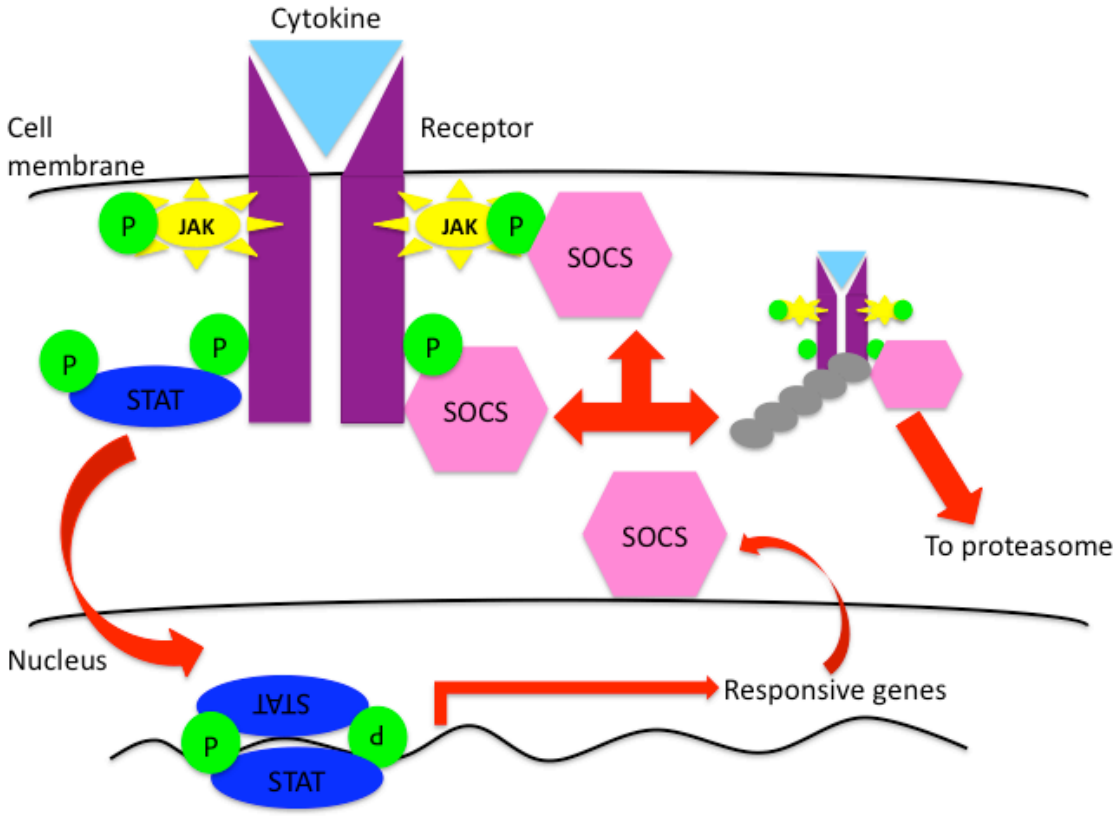

Figure 1-2. The JAK/STAT signaling pathway and its inhibition by the SOCS proteins. In this model, based on the current paradigm of JAK/STAT signaling, after the cytokine binds to the receptor, the receptor-associated JAKs activate and phosphorylate each other and the receptor. This allows for the STATs to bind and be phosphorylated. The phosphorylated STATs dimerize and travel to the nucleus where they induce the expression of the responsive genes, including the negative inhibitors suppressors of cytokine signaling. The SOCS proteins then block cytokine signaling by binding to the JAKs and/or the receptor and inhibiting phosphorylation of the STATs, or by acting as an E3 ubiquitin ligase and targeting the complex for proteasomal degradation.

CHAPTER 2
MATERIALS AND METHODS

Characterization of the SOCS1 Mimetic Peptide

Peptide Synthesis

Peptides were synthesized by Mr. Mohammad S. Haider of the Johnson laboratory as previously described (Szente et al., 1994). Briefly, synthesis involved conventional fluorenylmethyloxycarbonyl chemistry using an Applied Biosystems 431A automated peptide synthesizer (Applied Biosystems, Carlsbad, CA). A lipophilic group (palmitoyl-lysine) for cell penetration was added to the N-terminus as a last step, using a semi-automated protocol (Thiam et al., 1999). Peptides were characterized by mass spectrometry and purified by HPLC by the Interdisciplinary Center for Biotechnology Research at the University of Florida. Lyophilized peptides were received pre-measured from Mr. Haider upon request and they were then dissolved in DMSO or PBS prior to use (Sigma-Aldrich, St Louis, MO). The sequences of the peptides used in this study are presented in Table 3-1.

Binding Assays

Prior to performing the binding assays, we assessed the specificity of the anti-SOCS1-KIR antibody using an ELISA. The peptides SOCS1-KIR, SOCS1-KIR2A, and MuIFNγ(95-125) were bound to a 96-well plate at 3 µg/well in binding buffer (0.1M sodium carbonate and sodium bicarbonate, pH 9.6). After washing three times with wash buffer (0.9% sodium chloride and 0.05% Tween 20 in PBS), wells were blocked for 1 hour in blocking buffer (2% gelatin and 0.05% Tween 20 in PBS). Wells were washed again three times with wash buffer, and increasing concentrations of serum obtained from rabbits immunized with KLH-conjugated SOCS1-KIR (GenScript,

Piscataway, NJ) was added to the wells. After a 1 hour incubation period, the wells were washed 5 times and goat anti-rabbit IgG conjugated to horse radish peroxidase (HRP) was added. Following a 30 minute incubation, wells were washed and the HRP substrate *o*-phenylenediamine (OPD) in stable peroxidase buffer was added (Pierce, Rockford, IL). The reaction was stopped by the addition of 2N H_2SO_4. Absorbance was measured at 490nm using a 450-microplate reader (BioTek, Winooski, VT).

Binding assays were then performed as previously described (Ahmed et al., 2009). Briefly, peptides corresponding to the autophosphorylation loops of JAK2 and TYK2 were bound to a 96-well plate at 3 µg/well in binding buffer (0.1M sodium carbonate and sodium bicarbonate, pH 9.6). After washing three times with wash buffer (0.9% sodium chloride and 0.05% Tween 20 in PBS), wells were blocked for 1 hour in blocking buffer (2% gelatin and 0.05% Tween 20 in PBS). Wells were washed again three times and increasing concentrations of SOCS1-KIR dissolved in blocking buffer were added. The plate was incubated for 1 hour at room temperature, washed as described, and bound SOCS1-KIR was detected using rabbit anti-SOCS1-KIR antiserum (GenScript, Piscataway, NJ). Following a one-hour incubation, wells were washed, secondary antibody was added and the plate was incubated for 30 minutes. After the final wash steps, the HRP substrate OPD (Pierce, Rockford, IL) in stable peroxidase buffer was added, and the chromogenic reaction was stopped by the addition of 2N H_2SO_4. Absorbance was measured at 490nm using a 450-microplate reader (BioTek, Winooski, VT).

Splenocyte Proliferation Assay Using *Staphylococcal* Enterotoxin B

Spleens were harvested from naive SJL/J mice, and cells were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. SOCS1-KIR and SOCS1-KIR2A were

added at 0, 3.7, 33, and 100 μM concentrations and cells were incubated at 37°C, 5% CO2, for 2 hours to allow for cell penetration of the peptides. SEB (0.5 μg/mL) was added to each well and cells were incubated for 72 hours before proliferation was assessed using the CellTiter 96 AQueous One Cell Proliferation Assay (Promega, Madison, WI).

Peptide Toxicity Assay

Spleens were harvested from naive SJL/J mice, and cells were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. SOCS1-KIR and SOCS1-KIR2A were added at 0, 3.7, 33, and 100 μM concentrations and cells were incubated at 37°C, 5% CO2, for 72 hours. Proliferation was assessed using the CellTiter 96 AQueous One Cell Proliferation Assay (Promega, Madison, WI).

Inhibition of STAT Phosphorylation

Analysis of the inhibition of interferon-induced STAT phosphorylation was performed by Ms Rea Dabelic of the Johnson laboratory as previously described (Ahmed et al., 2009). Briefly, L929 fibroblasts were seeded at $1x10^6$ cells/mL in EMEM (10% FBS) in 6-well plates and incubated overnight at 37°C, 5% CO_2 to achieve confluency. The control peptide MuIFNGR1 (253-287), SOCS1-KIR, and Tkip were added at 12 and 24 μM concentrations and cells were incubated an additional 24 hours to allow for cell penetration of the peptides. Interferon γ (4000 u/mL) or IFNτ (10,000 u/mL) was added and cells were incubated for 2 hours. Following incubation, the cells were washed with cold PBS and harvested in radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentrations were analyzed using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). Proteins were resolved with 12% sodium dodecyl

sulfate-polyacrylimide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and probed with anti-pSTAT1 or anti-pSTAT3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then stripped and re-probed with anti-STAT1 or anti-STAT3 antibodies to determine if proteins were loaded equally. For STAT2 phosphorylation, WISH cells were used in lieu of L929 due to the availability of reagents. After treatment, the WISH cells were lysed and analyzed as above. Membranes were probed with anti-pSTAT2 and anti-STAT2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

SOCS-1 Knockout Mice Breeding and Treatment Protocols

The Institutional Animal Care and Use Committee (IACUC) at the University of Florida approved all of the animal protocols presented herein. Following the guidelines presented by IACUC and the Animal Care Services department at the University of Florida, euthanasia of neonates was performed by CO_2 asphyxiation for 10 to 15 minutes and death was confirmed with cervical dislocation. SOCS-1 heterozygous mice on a C57BL/6 genetic background (St. Jude Children's Hospital, Memphis, TN) were bred in to produce SOCS-$1^{+/+}$, SOCS-$1^{+/-}$, and SOCS-$1^{-/-}$ mice. Genotyping of the mice was performed by members of the Larkin laboratory using tail clips taken pre-weaning. Briefly, DNA was isolated from the tail clips using the DNeasy Blood, Tissue and Plant Kit from Qiagen (Valencia, CA). Quantitative PCR was then performed to assess the amount of SOCS-1 DNA was present in each sample, using the following primers: *SOCS-1* forward, 5'-GACACTCACTTCCGCACCTT-3', reverse, 5'-GGAAGGCACAGAATGCT-3' (Park et al., 2009); *actin* forward, 5'-CCTTCCTTCTTGGGTATGGA-3', reverse, 5'GGAGGAGCAATGATCTTGAT-3'. Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). qPCR

was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and a DNA Engine thermocycler equipped with a Chromo4 Continuous Fluorescence Detection system and OpticonMonitor Software (Bio-Rad Laboratories, Hercules, CA) with the following cycle conditions: 40 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 10 seconds (Park et al., 2009). Fluorescence was measured at 72°C.

Beginning one day after birth, prior to genotype analysis, pups remained untreated, received daily intraperitoneal (i.p.) injections with 10 µg/g SOCS1-KIR in PBS, received biweekly i.p. injections of 500,000 $CD4^+$ T cells, or received both treatments. $CD4^+$ T cells for adoptive transfer were isolated from the spleens and lymph nodes of wild-type C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) using the $CD4^+$ T cell Isolation Kit from Miltenyi Biotec, Inc. (Auburn, CA). Adoptive transfers were performed by Ms Erin Collins of the Larkin laboratory. Pups were weighed daily, monitored for survival, and observed for signs of disease such as weight loss, patchy fur, and lethargy. The number of pups of each genotype and in each treatment group is presented in the keys for the appropriate figures.

EAE

Mice for Studies Involving Experimental Allergic Encephalomyelitis

The Institutional Animal Care and Use Committee (IACUC) at the University of Florida approved all of the animal protocols presented herein. Female SJL/J mice (6 to 8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in standard SPF facilities. Following the guidelines laid out by IACUC and the Animal Care Services department at the University of Florida, euthanasia was performed by

CO_2 asphyxiation for 5 to 10 minutes before death was confirmed with cervical dislocation.

Induction of EAE

On day 1, SJL/J mice were injected with 300 µg /mouse bovine myelin basic protein (Invitrogen, Carlsbad, CA) emulsified in Complete Freund's Adjuvant with 8 mg/mL H37Ra *Mycobacterium tuberculosis* (Sigma-Aldrich, St. Louis, MO) subcutaneously into two sites at the base of the tail, followed by an intraperitoneal injection of pertussis toxin (400 ng/mouse in PBS) (List Biological Laboratories Inc., Campbell, CA). On day 3, the pertussis toxin injection was repeated. Mice were monitored daily for signs of EAE and assigned staged of disease accordingly to the following scale: 0, normal; 1, loss of tail tone; 2, hind limb weakness; 3, paraparesis; 4, paraplegia; 5, moribund; and 6, death.

Administration of Peptides

Beginning on day 12 post-immunization, after lymphocyte infiltration of the CNS has occurred but before symptoms are observable, mice, in groups of 5, were administered the following peptides or treatments every other day via i.p. injection: PBS, SOCS1-KIR (60 µg/mouse), or SOCS1-KIR2A (60 µg/mouse). For two of the experiments described below, peptides were administered beginning day 11 post-immunization due to mice presenting loss of tail tone.

Analysis of mRNA Expression

Spleens, brains, and spinal cords were collected from naïve and MBP-sensitized SJL/J mice experiencing EAE at Stage 1. Monocytes were isolated from the CNS tissues using a Percoll gradient (Sigma-Aldrich, St. Louis, MO) as previously described (Beeton and Chandy, 2007). $CD4^+$ T cells were isolated from single cell suspensions

using the $CD4^+$ T cell Isolation Kit from Miltenyi Biotec (Auburn, CA). No $CD4^+$ T cells were isolated from the naïve CNS tissue. Samples labeled "nonCD4 cells" refer to cells collected from the column after the $CD4^+$ T cell fraction was collected. RNA was isolated from 1 x 10^6 cells per sample using the SV Total RNA Isolation Kit from Promega (Madison, WI) and cDNA was generated using the iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA). qRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using the following primers (IDT, Coralville, IA): *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* forward, 5'-CTGCCAAGTATGATGACATCAAGAA-3', reverse, 5'-ACCAGGAAATGAGCTTGACA-3'; *SOCS-1* forward, 5'-GTGGTTGTGGAGGGTGAGAT-3', reverse, 5'-CCCAGACACAAGCTGCTACA-3; *SOCS-3* forward, 5'-AAGGGAGGCAGATCAACAGA-3', reverse, 5'-TGGGACAGAGGGCATTTAAG-3' (Takahashi et al., 2008). The qRT-PCR reaction was performed with a DNA Engine thermocycler equipped with a Chromo4 Continuous Fluorescence Detection System and OpticonMonitor software (Bio-Rad, Hercules, CA) with the following cycling conditions: initial enzyme activation at 95°C for 3 minutes, followed by 55 cycles of 95°C for 10 seconds, 60°C for 5 seconds, and 72°C for 15 seconds. Fluorescence was measured at 72°C. Expression of SOCS-1 and SOCS-3 mRNA was normalized to GAPDH mRNA expression and data are shown as fold expression relative to that of the naïve spleen $CD4^+$ T cells

Histology

Animals were sacrificed as described above on either day 12 or day 38 post-immunization with MBP and their brains were collected in 4% paraformaldehyde in PBS and fixed overnight. They were then stored in 70% ethanol until processing. With the help of Mr. Ken Lau of the Larkin laboratory and the Histology Resource Center at the

McKnight Brain Institute of the University of Florida, the brains were embedded in paraffin using a HISTOS 5 rapid microwave processor (Milestone Medical, Kalamazoo, MI) and Shandon Histocentre 3 embedding center (Thermo Fisher, Waltham, MA). The brains were cut anterior to lambda and the cerebrums were sliced into 5 µM thick sections (BrainInfo, 2007). Cut sections were stained with hematoxylin and eosin using SelecTech H&E reagents (SurgiPath Medical Ind., Inc., Richmond, IL) and the Autostainer XL (Leica Microsystems Inc., Bannockburn, IL). Slides were assessed with a Leica DM 2500 microscope (Leica Microsystems Inc., Bannockburn, IL) equipped with an Optronics color camera and MagnaFire software (Optronics, Goleta, CA). Two brains per treatment group were analyzed and the images presented are representative of the level of infiltration seen throughout the cerebrum.

Splenocyte Proliferation Assay Using Myelin Basic Protein

Spleens were harvested from MBP-immunized SJL/J mice at EAE stage 1. Splenocytes were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. SOCS1-KIR and SOCS1-KIR2A were added at 0, 3.7, and 11 µM concentrations and cells were incubated at 37°C, 5% CO_2, for 2 hours to allow for cell penetration. MBP (50 µg/mL) (Invitrogen, Carlsbad, CA) was then added to each well and cells were incubated for 72 hours before proliferation was assessed using the CellTiter 96 Aqueous One Cell Proliferation Assay (Promega, Madison, WI).

Detection of Basal IL-17A Production by Splenocytes from EAE Mice

SJL/J mice were immunized with MBP for EAE induction as described above and received i.p. injections of 100 µL PBS, SOCS1-KIR (60 µg/mouse), or SOCS1-KIR2A (60 µg/mouse) every other day beginning day 11 post-immunization. Treatments for these mice began day 11 instead of day 12 due to the appearance of symptoms in

some of the mice. Spleens were harvested from the mice 36 days post-immunization; all of the mice at this point were scored EAE Stage 1. Splenocytes were seeded at $5x10^6$ cells/well in RPMI (10% FBS) and incubated for 24 hours at 37°C, 5% CO_2. Supernatants were collected and analyzed for IL-17A by enzyme-linked immunosorbent assay using the IL-17A Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).

Detection of IL-17A Production by Splenocytes from EAE Mice in Response to MBP Stimulation

SJL/J mice were immunized with MBP for EAE induction as described above and had been receiving i.p. injections of 100 µL PBS, SOCS1-KIR (60 µg/mouse), or SOCS1-KIR2A (60 µg/mouse) every other day beginning on day 11 post-immunization. Treatments for these mice began day 11 instead of day 12 due to the appearance of symptoms in some of the mice. Spleens were harvested from the mice 27 days post-immunization with MBP; at this time the mice were all scored EAE Stage 1. Splenocytes were seeded at $5x10^6$ cells/well in RPMI (10% FBS) with or without 25 µg/mL MBP (Invitrogen, Carlsbad, CA) and incubated 24 hours at 37°C, 5% CO_2. Supernatants were collected and analyzed by enzyme-linked immunosorbent assay for IL-17A production using the IL-17A Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).

Peptide Inhibition of IL-17A Production by Splenocytes from EAE Mice in Response to MBP Stimulation

SJL/J mice were immunized with MBP for EAE induction as described above and were receiving 100 µL PBS every other day. Spleens were harvested 36 days post-immunization when the mice were scored at EAE Stage 1. Splenocytes were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. SOCS1-KIR and SOCS1-KIR2A were added at 0, 3.7, 11, and 33 µM concentrations and cells were incubated at 37°C,

5% CO_2 for 2 hours to allow for cell penetration of the peptides. MBP (Invitrogen, Carlsbad, CA) was then added to each well at 50 µg/mL and the cells were incubated an additional 24 hours. Supernatants were collected and analyzed for IL-17A by enzyme-linked immunosorbent assay using the IL-17A Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).

Inhibition of IL-17A and IFNγ Production by Splenocytes from EAE Mice in Response to IL-23

SJL/J mice were immunized with MBP for EAE induction as described above and were treated every other day with 100 µL PBS i.p. Mice were scored at EAE Stage 1. Spleens were harvested 37 days post-immunization. Splenocytes were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. SOCS1-KIR and SOCS1-KIR2A were added at 0, 3.7, 11, and 33 µM concentrations and the cells were incubated at 37°C, 5% CO_2, for 2 hours to allow for cell penetration. IL-23 (10 ng/mL) (eBioscience, San Diego, CA) was added to each well and the cells were incubated an additional 48 hours. Supernatants were collected and analyzed for IL-17A or IFNγ by enzyme-linked immunosorbent assay using the IL-17A or IFNγ Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).

Inhibition of IL-23 and IL-17A Induced STAT3 Activation

Analysis of the inhibition of IL-23 and IL-17A induced activation of STAT3 was performed by Ms Rea Dabelic of the Johnson laboratory using techniques similar to those described above for analyzing interferon-induced activation of STATs. Briefly, splenocytes isolated from MBP-immunized SJL/J mice experiencing EAE (Stage 1), were treated with SOCS1-KIR or SOCS1-KIR2A at 12 and 24 µM for 2 hours to allow for cell penetration, followed by incubation with IL-23 (10 ng/ml) (eBioscience, San Diego,

CA) for 10 minutes or IL-17A (0.5 ng/ml) (R&D Systems, Minneapolis, MN) for 2 hours at 37°C, 5% CO_2. The cells were washed with cold PBS, lysed using RIPA lysis buffer with phosphatase and protease inhibitors (Santa Cruz Biotechnologies, Santa Cruz, CA), and the protein concentration was determined by the standard BCA assay (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane for immunoblotting with anti-pSTAT3 and anti-STAT3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Relative intensity of the bands was measured with ImageJ software (NIH).

Statistical Analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Statistical analysis for SOCS-$1^{-/-}$ mice survival was performed using the Kaplan-Meier survival curve and log-ranked test. For the remaining experiments, statistical analyses were performed using the Student's *t* test or analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test as indicated in the results and/or figure legends.

CHAPTER 3
RESULTS

Characterization of the SOCS-1 Mimetic Peptide

SOCS1-KIR Binds to the Autophosphorylation Sites of the Janus Kinases JAK2 and TYK2

SOCS-1 is known to interact with all four Janus kinases—JAK1, JAK2, JAK3, and TYK2 (Dalpke et al., 2008; Johnson and Ahmed, 2006). In order to take a closer look at how the kinase inhibitor region (KIR) of SOCS-1 is involved in this interaction, we designed a peptide composed of the SOCS1-KIR amino acid sequence and tested its ability to bind to peptides composed of the autophosphorylation loops of the Janus kinases JAK2 and TYK2 (Table 3-1). Previous work done in our laboratory using biotinylated forms of the JAK2 and TYK2 peptides showed that the original SOCS-1 mimetic peptide Tkip and the mimetic being studied here, SOCS1-KIR, binds to these peptides but the alanine-substituted mutant peptide SOCS1-KIR2A does not (Ahmed et al., 2009). SOCS1-KIR2A, unlike SOCS1-KIR, has two alanines replacing the SOCS-1 phenylalanine residues 56 and 59 that are considered critical for binding to the JAKs (Yasukawa 1999). Using an antibody raised to KLH-conjugated SOCS1-KIR, which is specific for SOCS1-KIR but not SOCS1-KIR2A (Figure 3-1A), we were able to confirm that the peptide SOCS1-KIR binds strongly to both JAK2 and TYK2 when compared to the control peptide MuIFNγ(95-125) (Figure 3-1B).

SOCS1-KIR Inhibits SEB-Induced Proliferation of Splenocytes

Once we determined that the SOCS1-KIR peptide was able to interact with the Janus kinases JAK2 and TYK2, we wanted to determine if the peptide would mimic the action of SOCS-1 *in vitro*. The superantigen *Staphylococcal* enterotoxin B (SEB) is a powerful stimulant of splenocyte proliferation (Torres et al., 2001). Superantigens,

unlike traditional Th1 cell stimulating antigens, bind to the T cell receptor and major histocompatibility II (MHC II) molecule without the need for processing by the antigen presenting cell (Torres et al., 2001; Soos and Johnson, 1995). Interaction with the superantigen and MHC II activates the T cell receptor (TCR) signaling pathway, and induces T cells to produce large amounts of the mitogen IL-2, leading to their proliferation, and interferon γ (IFNγ) which activates and induces the proliferation of antigen presenting cells (Soos and Johnson, 1995; Cameron et al., 2001). SEB is known to increase the expression of SOCS-1 and SOCS-1 is known to inhibit immune cell proliferation in response to IL-2 and to block the signal transduction pathway of IFNγ (Chong et al., 2005; Plaza et al., 2004). Therefore, in order to determine if the SOCS1-KIR peptide imitates SOCS-1 activity in response to pro-proliferation signals, we treated splenocytes taken from naïve SJL/J mice with SOCS1-KIR or the alanine-substituted control peptide SOCS1-KIR2A for two hours prior to administering SEB to allow for the peptides to enter the cells. After an incubation period of 72 hours with the superantigen, cellular proliferation was measured using the CellTiter96 AQueous 1 Solution from Promega (Madison, WI). As can be seen in Figure 3-2, SOCS1-KIR, but not SOCS1-KIR2A, was able to significantly inhibit SEB-induced proliferation of a heterogeneous population of splenocytes.

SOCS1-KIR and the Alanine-Substituted Mutant Peptide SOCS1-KIR2A Are Not Cytotoxic

Previously, our laboratory had determined that the anti-proliferative effects of SOCS1-KIR and SOCS1-KIR2A were not due to any intrinsic cytotoxic properties of the peptides themselves by looking at the responses of the murine fibroblast cell line L929 incubated with these peptides for 24 hours (data not shown). However, it is possible

that the lack of proliferation in response to SEB seen above could be due to a cytotoxic effect that becomes observable with a longer incubation time such as the one used above. Therefore, we treated splenocytes from naïve SJL/J mice with increasing concentrations of these two peptides, incubated the cells for 72 hours as in the above assay, and assessed proliferation. Based on the results shown in Figure 3-3, we were able to determine that neither SOCS1-KIR nor SOCS1-KIR2A are cytotoxic, as there was no significant difference in proliferation between the untreated cells and the peptide treated cells.

SOCS1-KIR Inhibits STAT Phosphorylation in Response to Type I and Type II Interferons

As shown above, we have determined that the peptide SOCS1-KIR interacts with the activation loops of JAK2 and TYK2, and that SOCS1-KIR inhibits cellular proliferation in response to superantigen stimulation, but it is not cytotoxic. To take a closer look at how SOCS1-KIR interacts with the JAK/STAT signaling pathway, we decided to analyze the effect of SOCS1-KIR treatment on the phosphorylation of STAT proteins by these JAKs. The IFNγ receptor is known to use both JAK1 and JAK2 for STAT activation, while the IFNτ receptor, which is the same as that of the other type I interferons IFNα and IFNβ, uses JAK1 and TYK2. Therefore, we hypothesized that if SOCS1-KIR was binding to the autophosphorylation loops of JAK2 and TYK2 in cells like it does *in vitro*, than treatment with SOCS1-KIR would block the phosphorylation of the downstream STAT proteins by these JAKs. Accordingly, we treated the cell lines L929, a murine fibroblast cell line, and WISH, a human epithelial cell line, with SOCS1-KIR, the original SOCS-1 mimetic Tkip as a positive control, or the control peptide MuIFNGR1(257-283) for 24 hours to allow for cell penetration. Cells were then treated

with either the type II interferon, IFNγ, or the type I interferon, IFNτ, for 2 hours, and Western blots were performed using the protein extracted from the cell lysates. Membranes were probed with antibodies to the phosphorylated or unphosphorylated forms of STAT1, which is activated by both IFNγ and IFNτ, and STATs 2 and 3, which are known to be activated by IFNτ. As can be seen in Figure 3-4, SOCS1-KIR, like the original SOCS-1 mimetic peptide Tkip, was able to block the phosphorylation of these STAT proteins, while the control peptide, which in these experiments was the murine IFNγ receptor 1 (IFNGR1) peptide MuIFNGR1(253-287), had a reduced inhibitory effect on their phosphorylation. Thus, SOCS1-KIR can mimic at least three of the functions of SOCS-1, namely the interaction with the Janus kinases JAK2 and TYK2, inhibiting superantigen-induced cell proliferation, and inhibiting the downstream activation of interferon-induced STATs.

SOCS1-KIR Increases Survival and Enhances Development of SOCS-1$^{-/-}$ Mice When Combined with Adoptive Transfer of SOCS-1$^{+/+}$ CD4^{+} T Cells.

SOCS-1$^{-/-}$ mice suffer from a severe inflammatory disease that is characterized by multiple organ infiltration, stunted growth, and early death (Starr et al., 1998; Alexander et al., 1999; Zhang et al., 2001). To determine if the peptide could mimic the functions of SOCS-1 *in vivo*, we began injecting SOCS-1$^{-/-}$ mice, and their wild-type and heterozygous littermates, daily with 10 µg/g SOCS1-KIR in PBS intraperitoneally, beginning one day after birth. As can be seen in Figure 3-5A, SOCS1-KIR alone did not improve survival significantly over that of untreated SOCS-1$^{-/-}$ pups, but did lead to a significant increase in weight gained over the course of two weeks (Figure 3-5B). However, when combined with biweekly adoptive transfers of wild-type CD4^{+} T cells, in addition to significant increases in weight, 20% of pups survived past 60 days (Figure 3-

5A), which is comparable to the survival statistics seen in SOCS Box knockout mice (Zhang et al., 2001). This suggests that SOCS1-KIR is mimicking the activity of the SOCS-1 kinase inhibitory region *in vivo*, and that the peptide is capable of exerting a therapeutic effect in the face of a highly pro-inflammatory environment, such as the one seen in SOCS-$1^{-/-}$ mice.

EAE Mice

SOCS-1 mRNA Is Not Expressed by $CD4^+$ T Cells Infiltrating the CNS in EAE

Previous work in our laboratory using the tyrosine kinase inhibitory peptide, Tkip, has shown that the murine model of multiple sclerosis (MS), experimental allergic encephalomyelitis (EAE), is a good model with which to test the *in vivo* properties of SOCS-1 mimetic peptides (Mujtaba et al., 2005). Given the therapeutic efficacy of Tkip, we were first interested in determining the expression of endogenous SOCS-1 mRNA in $CD4^+$ T cells and non-$CD4^+$ monocytes found in the central nervous system (CNS) and in the spleen during active EAE. Because previous studies had shown that SOCS-3 mRNA expression is also relevant to the outcome of EAE (Berard et al., 2010; Stark et al., 2005; Qin et al., 2008), we also wanted to look at its expression in these cells. Accordingly, we performed quantitative real-time PCR (qRT-PCR) to determine SOCS-1 and SOCS-3 mRNA expression in CNS infiltrating $CD4^+$ T cells. The expression of SOCS-1 and SOCS-3 in splenic $CD4^+$ T cells was used for comparison since no $CD4^+$ T cells were isolated from the CNS of naïve mice. As can be seen in Figure 3-6, expression of both SOCS mRNAs in splenic cells from EAE mice increased slightly above that of $CD4^+$ T cells from the spleens of naïve mice. However, $CD4^+$ T cells isolated from the CNS of EAE mice did not express SOCS-1. Unlike SOCS-1 mRNA,

SOCS-3 mRNA expression increased approximately 6-fold over that of the naïve splenic $CD4^+$ T cells.

Because of the importance of other monocytic cells, such as microglia and macrophages, in the progression of EAE, we also looked at the expression of SOCS-1 and SOCS-3 mRNA in these cells. Non-$CD4^+$ splenic monocytes expressed low levels of SOCS-1 and SOCS-3 mRNA in both naïve and MBP-immunized mice. In the CNS, non-$CD4^+$ monocytes from EAE mice expressed higher levels of SOCS-1 mRNA than their splenic counterparts. The striking result, however, was the relatively high levels of SOCS-3 mRNA in these cells, which was approximately 60-fold greater than in naïve cells.

Thus, it appears that the dominant SOCS mRNA expression in the CNS of EAE mice is that of SOCS-3, not SOCS-1, and that this expression occurs primarily in the non-$CD4^+$ monocytes, not the infiltrating $CD4^+$ T cells. However, as discussed above, previous work has shown that the SOCS-1 mimetic Tkip has a therapeutic benefit in EAE (Mujtaba et al., 2005; Berard et al., 2010). Therefore, it is possible that augmenting SOCS-1 activity in the non-$CD4^+$ monocytes and supplying $CD4^+$ T cells with the SOCS-1 activity that they lack could be one of the mechanisms through which SOCS-1 mimetic peptides exert their effects.

SOCS1-KIR Prevents the Development of Severe EAE in SJL/J Mice Immunized with MBP

In order to determine if SOCS1-KIR, like Tkip, has a therapeutic effect in an autoimmune disease, we induced EAE in naïve SJL/J mice with an emulsion of 300 µg/mouse myelin basic protein (MBP) in Complete Freund's Adjuvant (CFA). Mice were injected with the MBP/CFA emulsion subcutaneously at the base of the tail, and also

received intraperitoneal injects of Pertussis toxin (400 ng/mouse) on days 1 and 3. Pertussis toxin has long been used in the induction of EAE in order to increase the activation and maturation state of antigen presenting cells, and recently has been determined to suppress that are activated in response to injection with myelin proteins (Chen et al., 2006). Twelve days post-immunization, treatment was initiated. We chose to begin treatment at this time because previous work in our laboratory had shown that infiltration of the central nervous system has already begun, even if the mice are not exhibiting symptoms. Mice received injections of 100 µL PBS, SOCS1-KIR (60 µg/mouse in PBS), or SOCS1-KIR2A (60 µg/mouse in PBS) intraperitoneally every other day. As can be seen in Figure 3-7, treatment with SOCS1-KIR dramatically decreased the incidence and severity of disease. All 20 mice in the PBS treated group experienced symptomatic disease, with 14 mice (70%) being classified stage 2 or higher at least one time during the course of the study (Table 3-2). In contrast, of the 15 mice included in the SOCS1-KIR treated group, 3 mice never experienced any observable symptoms, 5 mice were classified as stage 1 or below for the majority of the study, and 7 mice (47%) were classified as stage 2 or higher. Mice treated with the alanine-substituted control peptide, SOCS1-KIR2A, fared similarly to that of the PBS-treated mice; all 15 mice exhibited observable symptoms and 12 (80%) were classified as stage 2 or higher. For PBS treated mice, the maximum average disease severity was 4.2, while for SOCS1-KIR treated mice it was 1.8. As with the incidence of disease, the maximum average disease severity for SOCS1-KIR2A treated mice at a score of 3.4 was similar to that of the PBS treated group.

SOCS1-KIR Reverses and Inhibits Infiltration of the CNS by Leukocytes in EAE

One of the hallmarks of both MS and EAE is disruption of the blood-brain barrier leading to infiltration of the central nervous system by auto-reactive lymphocytes. Based on the lower severity of disease seen in mice treated with SOCS1-KIR, we hypothesized that fewer immune cells would be present in the CNS of these mice. Brains were thus collected from mice in the different treatment groups 37 days post-immunization. After fixation, the brains were embedded in paraffin, sectioned, and stained with H&E. While the PBS-treated and SOCS1-KIR2A treated control groups had signs of extensive leukocyte infiltration into the brain, the SOCS1-KIR treated mouse had no visible infiltrations at this time point (Figure 3-8A, B, and C). Interestingly, as shown in Figure 3-8D, cellular infiltration of the CNS has already begun at day 12, as compared to the naïve mouse brain (Figure 3-8E), before treatment with the peptides was initiated. Therefore, not only does treatment with SOCS1-KIR block further infiltration into the CNS, it also reverses the appearance of any infiltrates that had been present prior to treatment.

SOCS1-KIR Inhibits MBP-Induced Splenocyte Proliferation in EAE Mice

While our initial characterization of the SOCS1-KIR peptide looked at non-specific proliferation of splenocytes using SEB, we wanted to determine if the peptide would also inhibit antigen-specific cellular proliferation in EAE. Therefore, 37 days post-immunization, spleens were harvested from PBS-treated mice and the cells were isolated and then pre-treated with SOCS1-KIR or the alanine-substituted control peptide for two hours to allow for cell penetration, prior to the addition of MBP (50 µg/mL). After 72 hours, proliferation was assessed as above. SOCS1-KIR significantly inhibited MBP-induced splenocyte proliferation, while the control peptide did not (Figure 3-9).

These results suggest that one way in which SOCS1-KIR is able to inhibit the development of severe EAE is by blocking the proliferation of auto-reactive cells in a heterogeneous population.

Th17 Cells

SOCS1-KIR Inhibits IL-17A Production by Splenocytes of Mice Immunized with MBP

Two of the cytokines that are most often linked to the development and severity of MS and EAE are interleukin-17A and IFNγ. Therefore, we wanted to determine what the basal levels of production of IL-17A by splenocytes isolated from the different treatment groups are, and whether treatment with SOCS1-KIR alters the production of this cytokine. As shown in Figure 3-10, after 24 hours in culture, basal levels of production of IL-17A were decreased by approximately 6-fold in the mice treated with SOCS1-KIR as compared to the PBS-treated group. Splenocytes from SOCS1-KIR2A-treated mice also had reduced IL-17A production, but not to the extent of that seen with SOCS1-KIR. Therefore, these preliminary results suggest that treatment with SOCS1-KIR alters the basal level of production of one of the key cytokines of EAE.

After determining that splenocytes from SOCS1-KIR treated mice were producing less IL-17A in culture than those from the control mice, we wanted to see if that pattern would hold true if the cells were stimulated with exogenously supplied antigen. Therefore, MBP (25 µg/mL) was added to the cells and cytokine production was measured after 24 hours of incubation. In Figure 3-11, it can be seen that even when presented with MBP, the splenocytes from the SOCS1-KIR treated mice produced roughly 8-fold less IL-17A than the PBS treated mice. Consistent with the results above, splenocytes from SOCS1-KIR2A treated mice produced twice as much IL-17A in

response to MBP stimulation as those from SOCS1-KIR treated mice. These experiments show that not only does treatment with the SOCS-1 mimetic peptide inhibit production of basal levels of IL-17A by splenocytes from MBP-immunized mice, but that it also inhibits those splenocytes from strongly responding even when the antigen is present.

SOCS1-KIR Inhibits MBP-Induced IL-17A Production by Splenocytes from EAE Mice

While we know that splenocytes from mice treated with SOCS1-KIR produce less IL-17A than those from control mice, even with MBP stimulation, we wanted to determine if the peptide could affect the levels of IL-17A produced by Th17 cells from untreated mice. Accordingly, splenocytes were isolated from MBP-immunized mice and treated with SOCS1-KIR or the control peptide for two hours before the addition of MBP (50 µg/mL), and IL-17A production was measured 24 hours later. As seen in Figure 3-12, SOCS1-KIR, at 33 µM, inhibited MBP-induced IL-17A production more than the control peptide. Thus, SOCS1-KIR was able to inhibit the activity of antigen-sensitized Th17 cells in culture.

SOCS1-KIR Inhibits IL-23 Induced IL-17A and IFNγ Production by Splenocytes from EAE Mice

IL-23, as mentioned above, is known to play a role in the maintenance of the Th17 cell phenotype and their activity. We consequently wanted to determine if SOCS1-KIR would have an effect on IL-23 stimulation of splenocytes from MBP-immunized mice. First, we looked at IL-17A production by splenocytes after treatment with IL-23. Splenocytes from MBP-immunized mice were treated with SOCS1-KIR or the control peptide for two hours followed by the addition of IL-23 (10 ng/mL). Cells were incubated for 48 hours before IL-17A levels were quantified as above. As seen in Figure 3-13A,

SOCS1-KIR was able to block IL-23-induced IL-17A production to the levels seen in unstimulated cells.

In addition to producing IL-17A, a subset of Th17 cells that can also produce IFNγ has also been described, and recent studies have shown that these IFNγ^{+} Th17 cells preferentially accumulate in the CNS of mice with EAE and in patients with MS (Kebir et al., 2009). Therefore, we also wanted to determine whether or not SOCS1-KIR could inhibit the IL-23 stimulated production of IFNγ. Accordingly, splenocytes from MBP-immunized mice were treated with the peptides, as described above, prior to the addition of IL-23, and IFNγ production was assessed after 48 hours. As can be seen in Figure 3-13B, IFNγ production is significantly increased by IL-23 stimulation, but treatment with SOCS1-KIR was able to inhibit this production, even at 11 μM concentration. SOCS1-KIR2A, consistent with the results shown throughout this study, was less effective at inhibiting IFNγ production in response to IL-23. Thus, SOCS1-KIR can inhibit IL-23-induced pro-inflammatory cytokine production by splenocytes from EAE, which may contribute to its therapeutic effects against EAE.

SOCS1-KIR Inhibits IL-23 Induced STAT3 Phosphorylation

IL-23 is known to signal via the JAK/STAT pathway utilizing the Janus kinases JAK2 and TYK2, and previous work has shown that it is regulated by SOCS-3 (Langrish et al., 2004; Watford et al., 2004). Since we determined that SOCS1-KIR can inhibit the ability of splenocytes to respond to IL-23, as per the production of the cytokines IL-17A and IFNγ, and we know that SOCS1-KIR binds to both JAK2 and TYK2, and can inhibit STAT phosphorylation by these Janus kinases in response to interferon stimulation, we decided to determine the ability of SOCS1-KIR to inhibit IL-23 induction of phosphorylation of STAT3 (pSTAT3) in splenocytes from mice with active EAE. As

shown in Figure 3-14, cells from these mice had constitutively phosphorylated STAT3, possibly as a result of ongoing disease. This phosphorylation was enhanced approximately 2-fold by treatment with IL-23 (10 ng/ml). SOCS1-KIR at 24 μM was able to inhibit IL-23 activation of STAT3 to the level of that seen in untreated cells, while SOCS1-KIR2A had no effect. Thus, SOCS1-KIR inhibits IL-23 function at the level of signal transduction by blocking the ability of the JAKs to phosphorylate STAT3

In contrast to IL-23, the signaling pathway used by IL-17A is still being delineated. Much of the work to date has focused on the ability of IL-17A stimulation to activate mitogen-activated protein kinase (MAPK) pathways and NF-κB, and on the similarity of the proximal signaling molecules of the IL-17A pathway, such as TRAF6, to those found in the toll-like receptor (TLR) and IL-1 signaling pathways (Shen and Gaffen, 2008; Gaffen 2008; Moseley et al., 2003; Korn et al., 2009); however, there is evidence that the JAK/STAT pathway is involved (Subramaniam et al., 1999). Therefore, to determine if IL-17A induces STAT3 phosphorylation, and if SOCS1-KIR can block that phosphorylation, splenocytes from EAE mice were treated with IL-17A (0.5 ng/ml) for 2 hours. As can be seen in Figure 3-14, IL-17A did not enhance the constitutive STAT3 phosphorylation present in these cells. However, SOCS1-KIR, at 24 μM, did reduce the pSTAT3 level by approximately half, while SOCS1-KIR2A had no effect.

Table 3-1. Peptide sequences used and/or discussed in this study.

Peptide	Sequence
SOCS1-KIR	^{53}DTHFRTFRSHSDYRRI
SOCS1-KIR2A	^{53}DTHFATFASHSDYRRI
JAK2	^{1001}LPQDKEYYKVKEP
TYK2	VPEGHEYYRVRED
Tkip	WLVFFVIFYFFR
Tkip2A	WLVFFVIAYFAR
MuIFNGR1(253-287)	^{253}TKKNSFRKSIMLPKSLLSVVKSATLETKPESKYS
MuIFNγ(95-125)	^{95}AKFEVNNPQVQRQAFNELIRVVHQLLPESSL

Modifications were made to the peptides depending on the experiment being performed. When working in cell culture or mice, the peptides were synthesized with a palmitic acid added for cell penetration.

Table 3-2. Summary of disease incidence and severity in SOCS1-KIR treated mice versus control mice with EAE.

Treatment Group	N	% Disease Incidence	% Incidence of Disease Above Stage 2	Maximum Average Disease
PBS	20	100	70	4.2
SOCS1-KIR	15	80	47	1.8
SOCS1-KIR2A	15	100	80	3.4

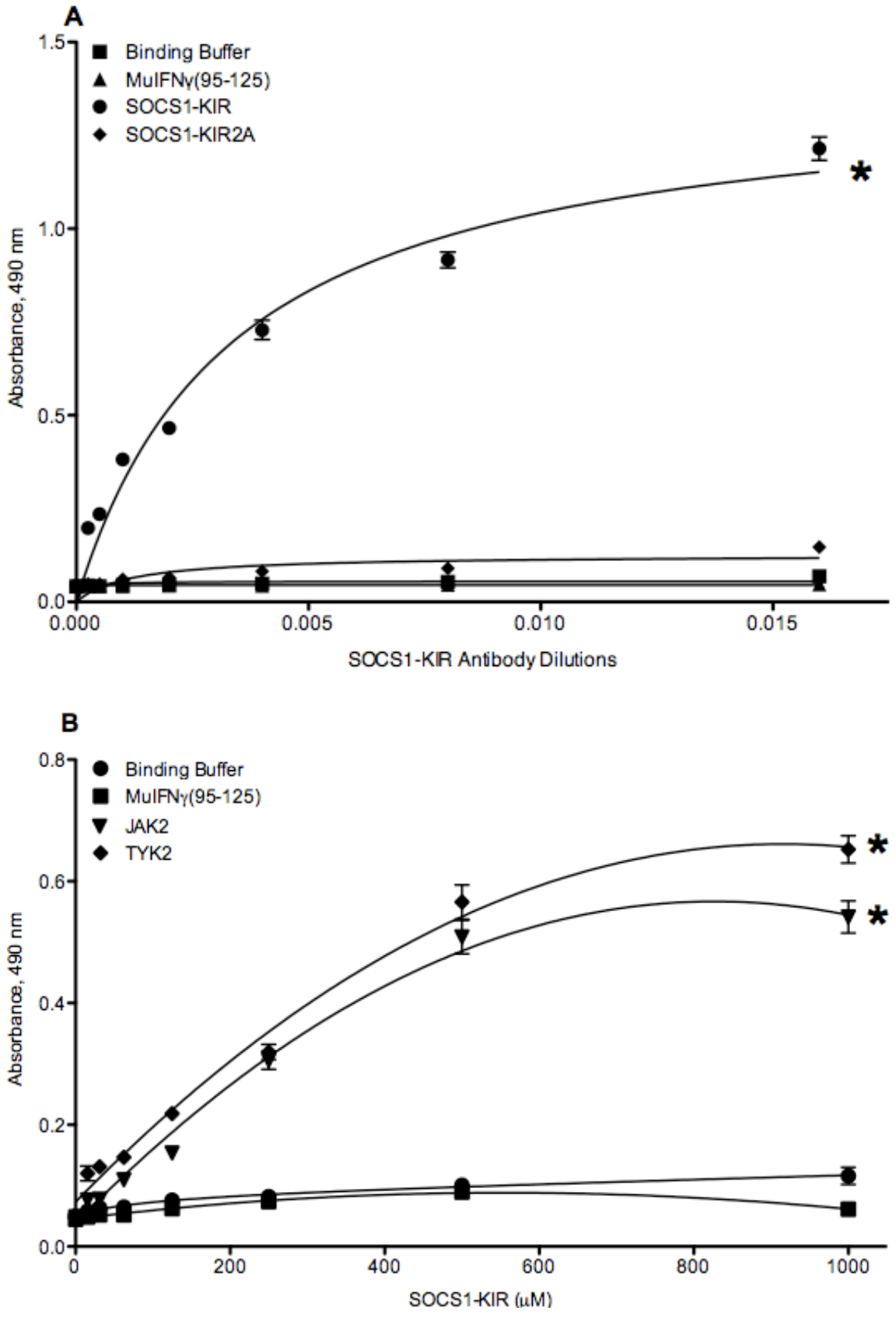
A
Binding Buffer
MuIFNγ(95-125)
SOCS1-KIR
SOCS1-KIR2A
Absorbance, 490 nm
1.5
1.0
0.5
0.0
0.000
0.005
0.010
0.015
SOCS1-KIR Antibody Dilutions
*
B
Binding Buffer
MuIFNγ(95-125)
JAK2
TYK2
Absorbance, 490 nm
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
SOCS1-KIR (μM)
*
*

Figure 3-1. SOCS1-KIR binds to the Janus kinases JAK2 and TYK2. A) Anti-SOCS1-KIR antibody is specific for SOCS1-KIR. SOCS1-KIR, SOCS1-KIR2A, and MuIFNγ(95-125) were bound to a 96-well plate at 3 µg/well. After blocking, increasing concentrations of anti-SOCS1-KIR antiserum were added to each well. Bound antibody was detected using goat anti-rabbit IgG-HRP conjugate. Absorbance was measured at 490 nm with a standard plate reader. The binding of anti-SOCS1-KIR antibody to SOCS1-KIR* was significantly different that binding to either control peptides, as determined by two-way ANOVA (*P < 0.001). B) SOCS1-KIR binds to JAK2 and TYK2. JAK2, TYK2, and the control peptide MuIFNγ(95-125) were bound to a 96-well plate at 3 µg/well. After blocking, increasing concentrations of SOCS1-KIR were added. Bound SOCS1-KIR was detected with the anti-SOCS1-KIR antibody followed by goat anti-rabbit IgG-HRP conjugate. Absorbance was measured at 490 nm with a standard plate reader. The binding of SOCS1-KIR to JAK2* and TYK2* was significantly different than binding to the control peptide, as determined by two-way ANOVA (*P < 0.001). For parts A and B, the data are representative of three different experiments. Figure courtesy of R. Dabelic.

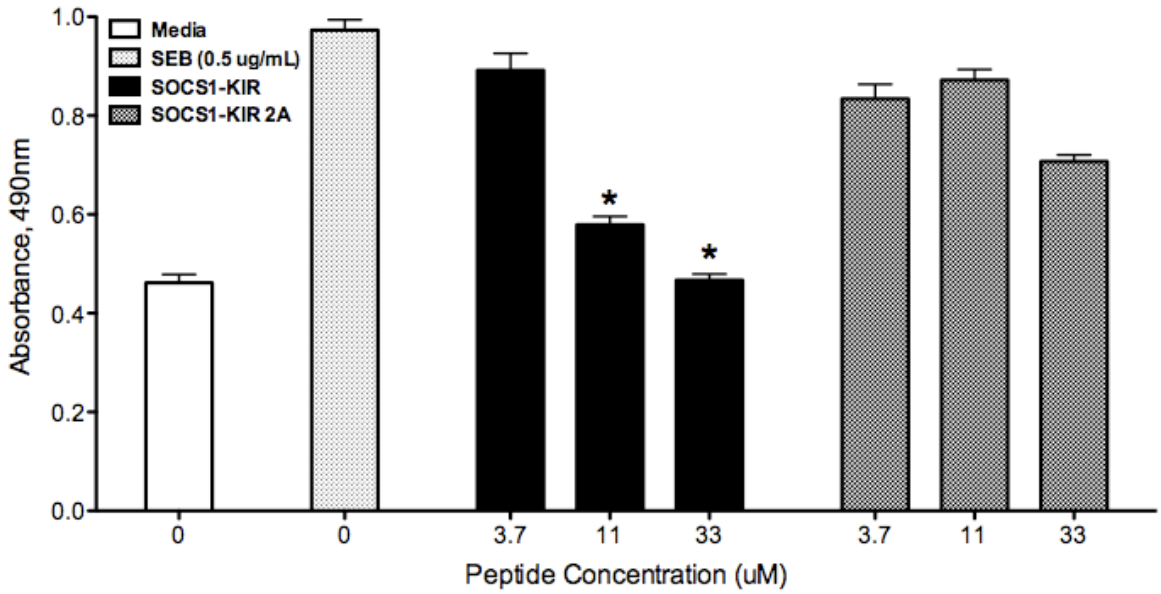

Figure 3-2. SOCS1-KIR inhibits SEB-induced splenocyte proliferation. Spleens were harvested from naive SJL/J mice, and the isolated splenocytes were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. Peptides were added at the above concentrations and cells were incubated for 2 hours at 37°C, 5% CO_2. SEB (0.5 µg/mL) was then added to each well and cells were incubated 72 hours before proliferation was assessed using the CellTiter 96 AQueous One Cell Proliferation Assay (Promega). Absorbance was read at 490nm. *$P < 0.001$ as determined by two-way ANOVA. The data is representative of five different experiments.

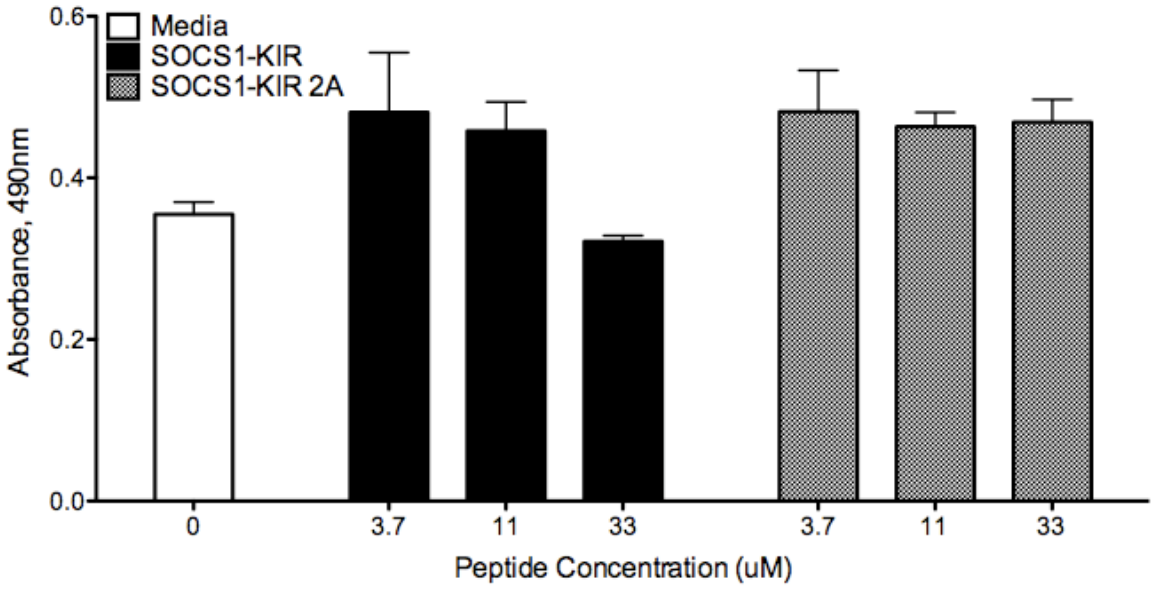

Figure 3-3. SOCS1-KIR is not cytotoxic. Spleens were harvested from naive SJL/J mice, and isolated cells were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. Peptides were added at the above concentrations and cells were incubated at 37°C, 5% CO_2, for 72 hours. Proliferation was assessed using the CellTiter 96 AQueous One Cell Proliferation Assay (Promega). Absorbance was measured at 490 nm. Statistics as performed with two-way ANOVA showed no significant difference. The data is representative of three separate experiments.

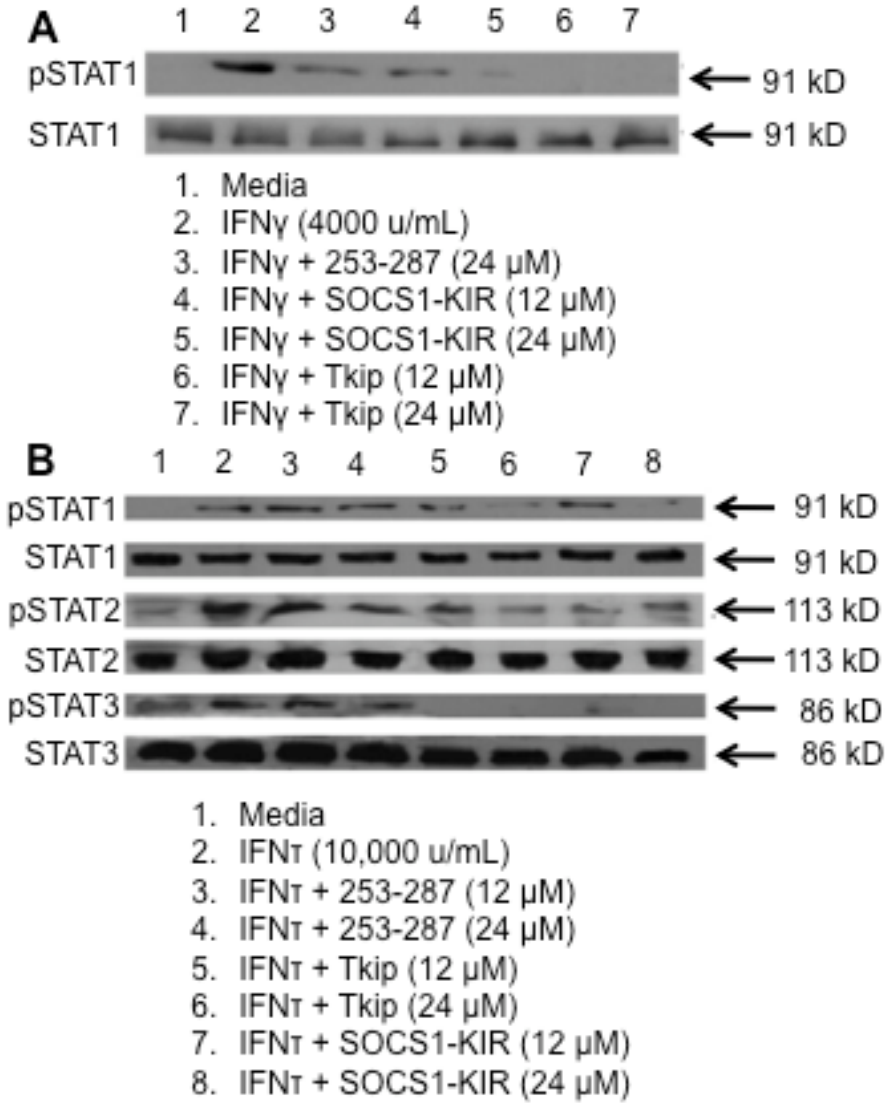

Figure 3-4. SOCS1-KIR inhibits IFNγ and IFNτ induced STAT phosphorylation. A) L929 cells were treated with the above peptides for 2 hours before IFNγ (4000 u/mL) were added to allow for cell penetration. After a two-hour incubation period, cells were lysed and protein concentration was determined by the standard BCA assay. Proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane and membranes were probed with the indicated antibodies. B) Cells were treated as above except with IFNτ (10,000 u/mL). For STAT2, WISH cells were used in lieu of L929 cells due to the availability of reagents. Figure courtesy of R. Dabelic.

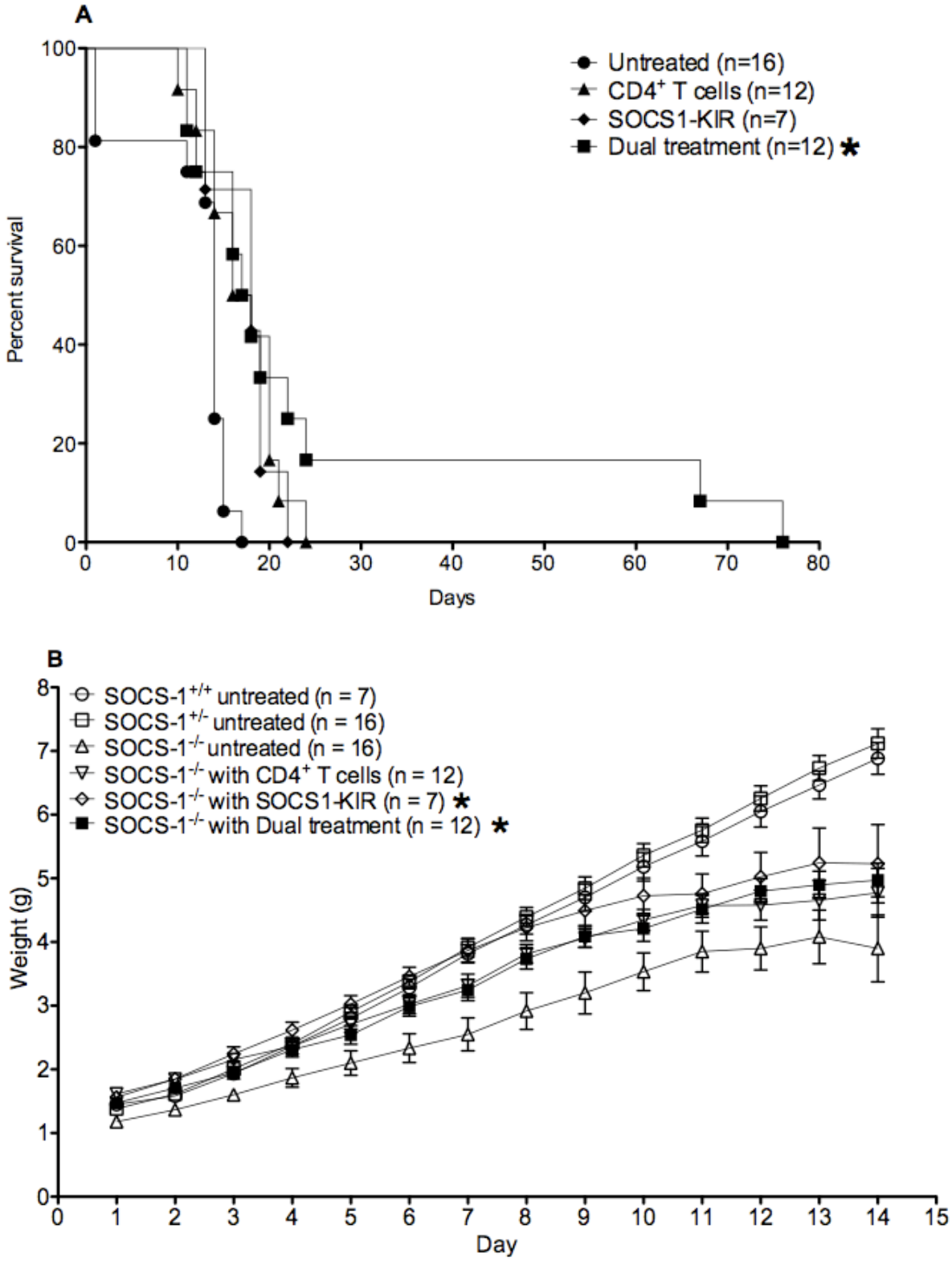
A
Untreated (n=16)
CD4+ T cells (n=12)
SOCS1-KIR (n=7)
Dual treatment (n=12) *
Percent survival
Days
B
SOCS-1+/+ untreated (n = 7)
SOCS-1+/- untreated (n = 16)
SOCS-1-/- untreated (n = 16)
SOCS-1-/- with CD4+ T cells (n = 12)
SOCS-1-/- with SOCS1-KIR (n = 7) *
SOCS-1-/- with Dual treatment (n = 12) *
Weight (g)
Day

Figure 3-5. SOCS1-KIR in combination with adoptive transfer of wild-type $CD4^+$ T cells enhances survival and growth of SOCS-$1^{-/-}$ mice. A) Survival of SOCS-$1^{-/-}$ mice. SOCS-$1^{-/-}$ mice remained untreated, or were injected i.p. with 10 µg/g SOCS1-KIR in PBS daily, 500,000 SOCS-$1^{+/+}$ $CD4^+$ T cells biweekly, or received both treatments, beginning one day after birth. The number of mice per group is presented in the figure key. Survival of mice in the dual treatment group was significantly longer compared to the untreated mice as determined by the Kaplan-Meier curve with log-rank test (*P = 0.01). B) Weights of untreated SOCS-$1^{+/+}$, untreated SOCS-$1^{+/-}$, and SOCS-$1^{-/-}$ mice undergoing the various treatments described in part A. The number of mice per group is presented in the figure key. SOCS-$1^{-/-}$ mice receiving SOCS1-KIR alone or the dual treatment were significantly heavier over the course of 14 days than untreated SOCS-$1^{-/-}$ mice, as determined by two-way ANOVA (*P < 0.05).

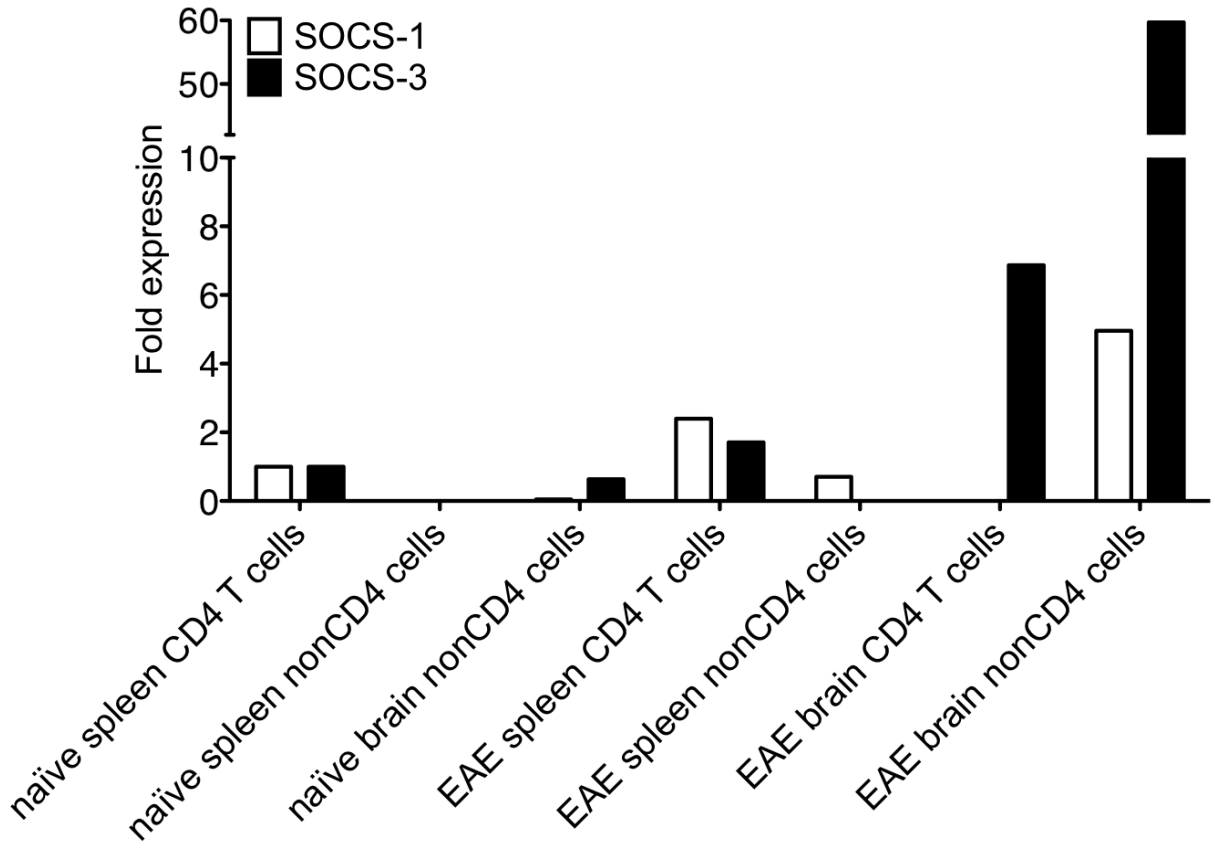

Figure 3-6. $CD4^+$ T cells infiltrating the CNS in EAE do not express SOCS-1 mRNA. Spleens, brains, and spinal cords were collected from naïve and MBP-sensitized SJL/J mice experiencing EAE at Stage 1. Monocytes were isolated from the combined CNS tissues of 3 mice using a Percoll gradient. CD4+ T cells were isolated from single cell suspensions of CNS infiltrating monocytes or splenocytes using the CD4+ T cell isolation kit (Miltenyi Biotec). No CD4+ T cells were isolated from the naïve CNS tissue. Samples labeled "nonCD4 cells" refer to cells collected from the column after the CD4+ T cell fraction was collected. Expression of SOCS-1 and SOCS-3 mRNA was normalized to GAPDH mRNA expression and data are shown as fold expression relative to the expression of each gene in the naïve spleen CD4+ T cells.

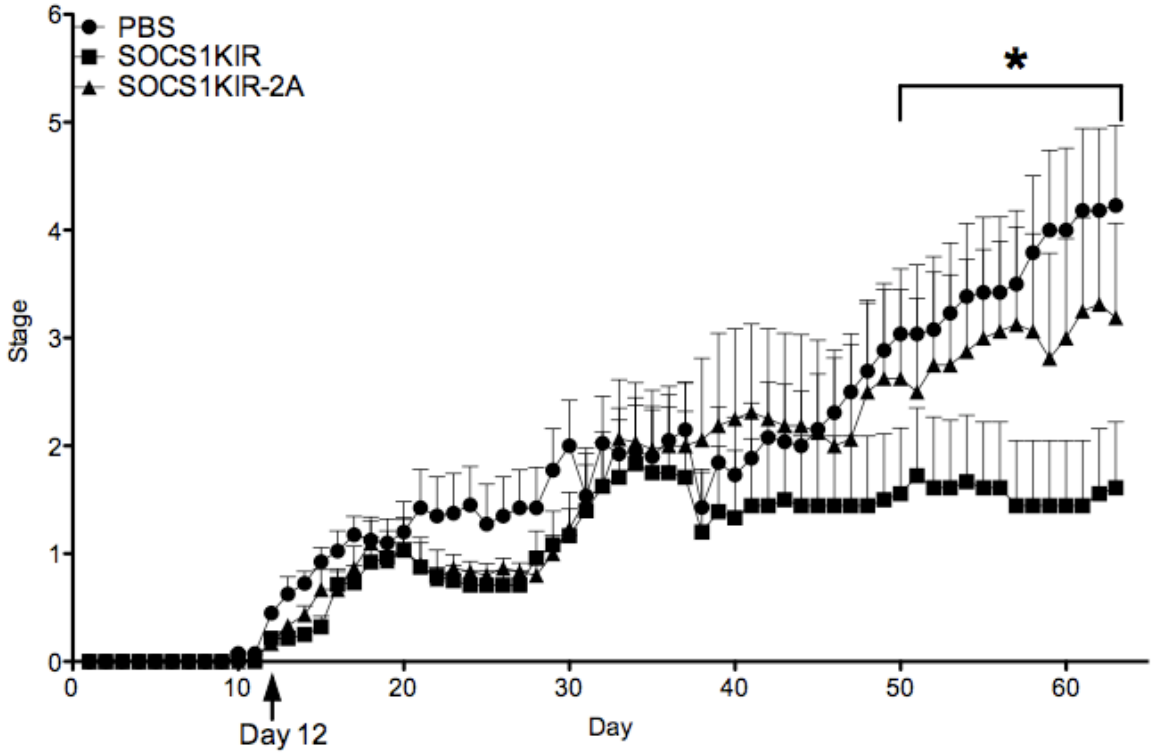

Figure 3-7. SOCS1-KIR protects mice from experimental autoimmune encephalomyelitis. SJL/J mice were injected i.p. with PBS, SOCS1-KIR 2A (60g/mouse), or SOCS1-KIR (60g/mouse) every other day starting 12 days post-immunization with MBP for EAE induction, after lymphocyte infiltration of the CNS. Mice were followed daily for signs of EAE, and the mean daily severity of disease was graded based upon the following scale: 0, no disease; 1, loss of tail tone; 2, hind leg weakness; 3, paraparesis; 4, paraplegia; 5, moribund; and 6, death. For PBS treated mice (n=20) the maximum average disease severity was 4.2; for SOCS1-KIR treated mice (n=15) the maximum average disease severity was 1.8; for SOCS1-KIR 2A treated mice (n=15) the maximum average disease severity was 3.4. The mean disease stage of each group was significantly different from that of the other groups beginning on day 52, as determined by paired Student's *t* test (*P < 0.0001).

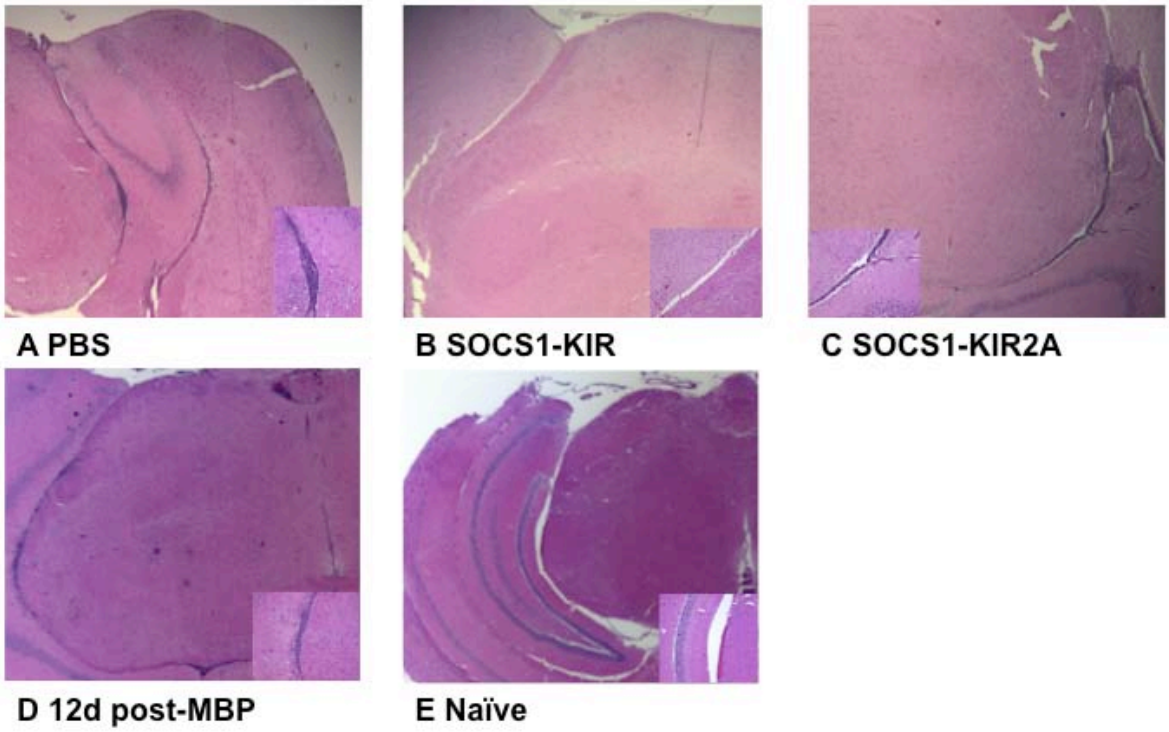

Figure 3-8. SOCS1-KIR reverses and prevents leukocyte infiltration of the CNS during EAE. A-C) SJL/J mice were injected with 100 µL (A) PBS, (B) SOCS1-KIR (60 µg/mouse), or (C) SOCS1-KIR2A (60 µg/mouse) every other day beginning 12 days post-immunization with MBP and sacrificed on day 38. D) The brain of an untreated mouse that was asymptomatic, 12 days post-immunization with MBP. E) The brain of a naïve SJL/J mouse. Brains were collected in 4% PFA in PBS and fixed overnight before being transferred to 70% ethanol. They were then embedded in paraffin, cut, and stained with H&E. Two brains per treatment group were analyzed and the above images are representative of the level of infiltration found throughout the cerebrum. Magnification is 1.25 x; each inset is 20 x.

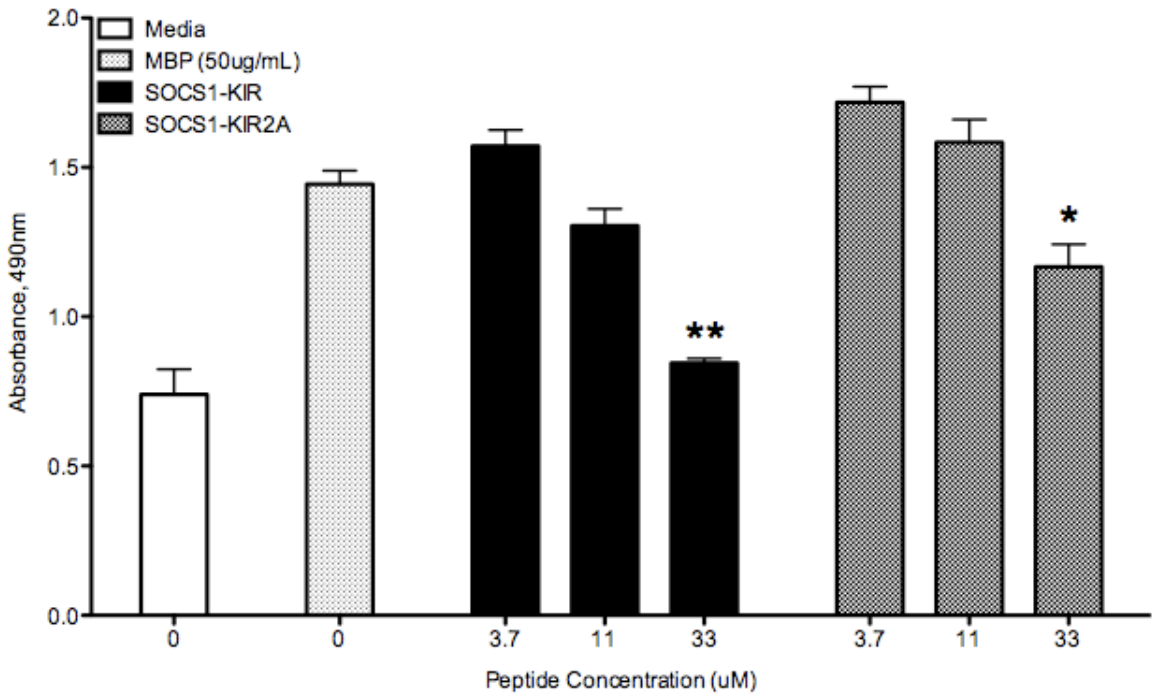

Figure 3-9. SOCS1-KIR inhibits MBP-induced proliferation of splenocytes. Spleens were harvested from MBP-immunized SJL/J mice (EAE stage 1) and isolated cells were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. Peptides were added at the above concentrations and cells were incubated at 37°C, 5% CO_2, for 2 hours. MBP (50 µg/mL) was then added to each well and the cells were incubated for 72 hours before proliferation was assessed using the CellTiter 96 AQueous One Cell Proliferation Assay (Promega). Absorbance was read at 490 nm. * $P < 0.01$, ** $P < 0.001$ as determined by two-way ANOVA. The data is representative of three separate experiments.

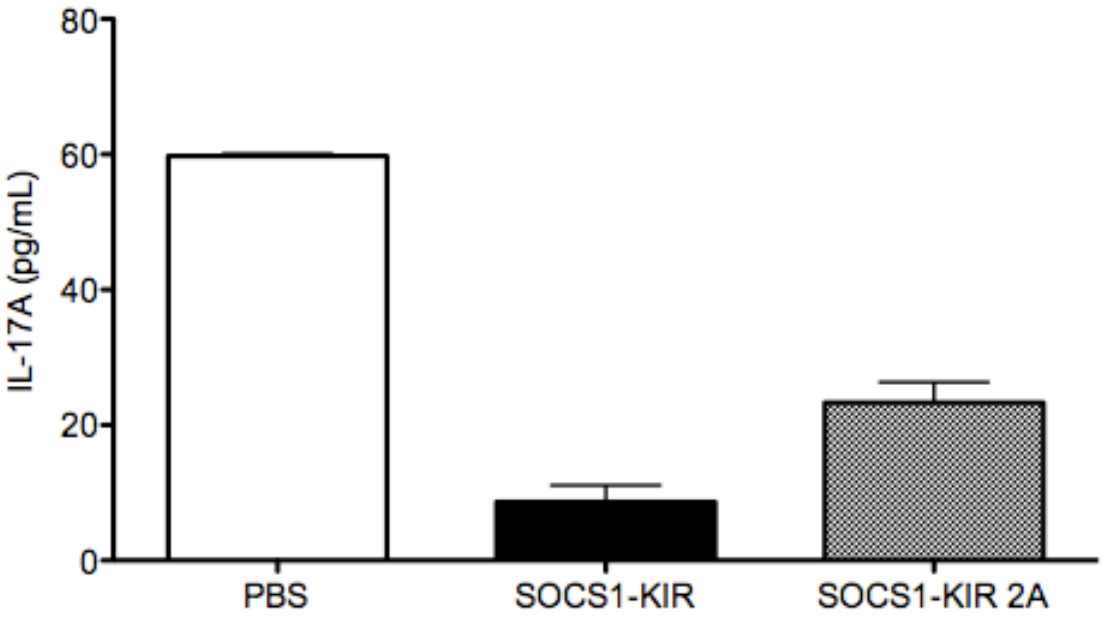

Figure 3-10. IL-17A production by splenocytes is inhibited in EAE by treatment with SOCS1-KIR. Splenocytes were isolated from SJL/J mice 36 days after immunization with MBP. Prior to harvesting the spleens, the mice had been receiving i.p. injections of 100 µL PBS, SOCS1-KIR (60 µg/mouse), or SOCS1-KIR2A (60 µg/mouse) every other day beginning on day 12 post-immunization, and were scored EAE stage 1. Cells were seeded at $5x10^6$ cells/well in RPMI (10% FBS) and incubated 24 hours at 37°C, 5%CO_2. Supernatants were collected and analyzed for IL-17A using the IL-17A Ready-Set-Go ELISA kit (eBioscience). Absorbance was measured at 490 nm. The assay was performed in triplicate and the data is representative of three separate experiments.

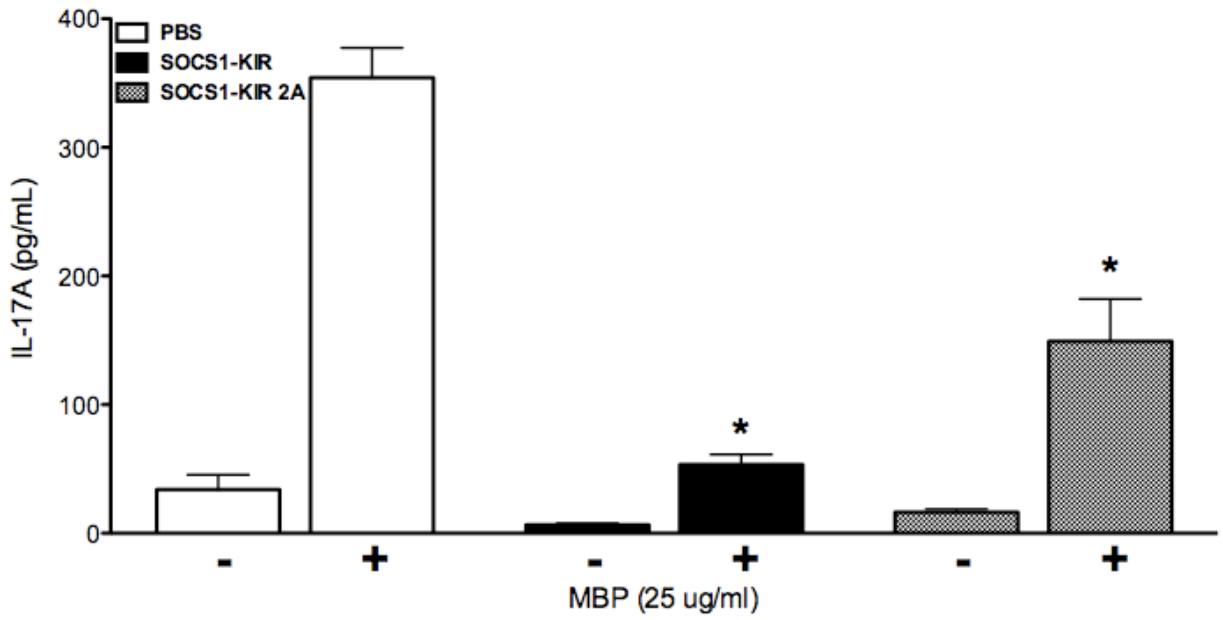

Figure 3-11. The ability of Th17 cells to respond to MBP is reduced in mice treated with SOCS1-KIR. Splenocytes were isolated from SJL/J mice 36 days after immunization with MBP. Prior to harvesting the spleens, the mice had been receiving i.p. injections of 100 µL PBS, SOCS1-KIR (60 µg/mouse), or SOCS1-KIR2A (60 µg/mouse) every other day beginning on day 12 post-immunization, and were scored EAE stage 1. Cells were seeded at $5x10^6$ cells/well in RPMI (10% FBS) with or without MBP (25 µg/mL) and incubated 24 hours at 37°C, 5%CO_2. Supernatants were collected and analyzed for IL-17A using the IL-17A Ready-Set-Go ELISA kit (eBioscience). Absorbance was measured at 490 nm. * $P < 0.001$ as determined by two-way ANOVA. The data is representative of three separate experiments.

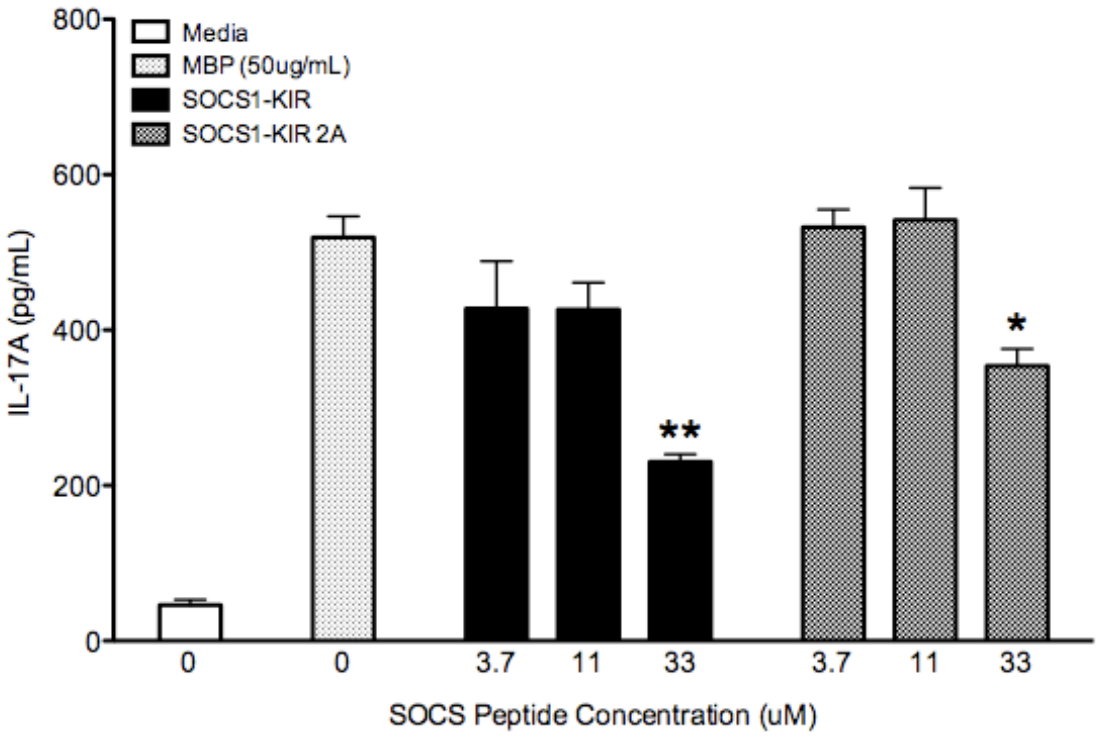

Figure 3-12. MBP-induced IL-17A production by splenocytes from EAE mice is inhibited by SOCS1-KIR. Splenocytes were isolated from SJL/J mice 36 days after immunization with MBP. Prior to harvesting the spleen, the mouse was scored at EAE stage 1. Cells were seeded at $5x10^6$ cells/well in RPMI (10% FBS) in a 96-well plate. Peptides were added at the above concentrations and cells were incubated at 37°C, 5% CO_2, for 2 hours. MBP (50 µg/mL) was then added to each well and the cells were incubated an additional 24 hours. Supernatants were collected and analyzed for IL-17A using the IL-17A Ready-Set-Go ELISA kit (eBioscience). Absorbance was read at 490 nm. * $P < 0.01$, ** $P < 0.001$ as determined by two-way ANOVA. The data is representative of three separate experiments.

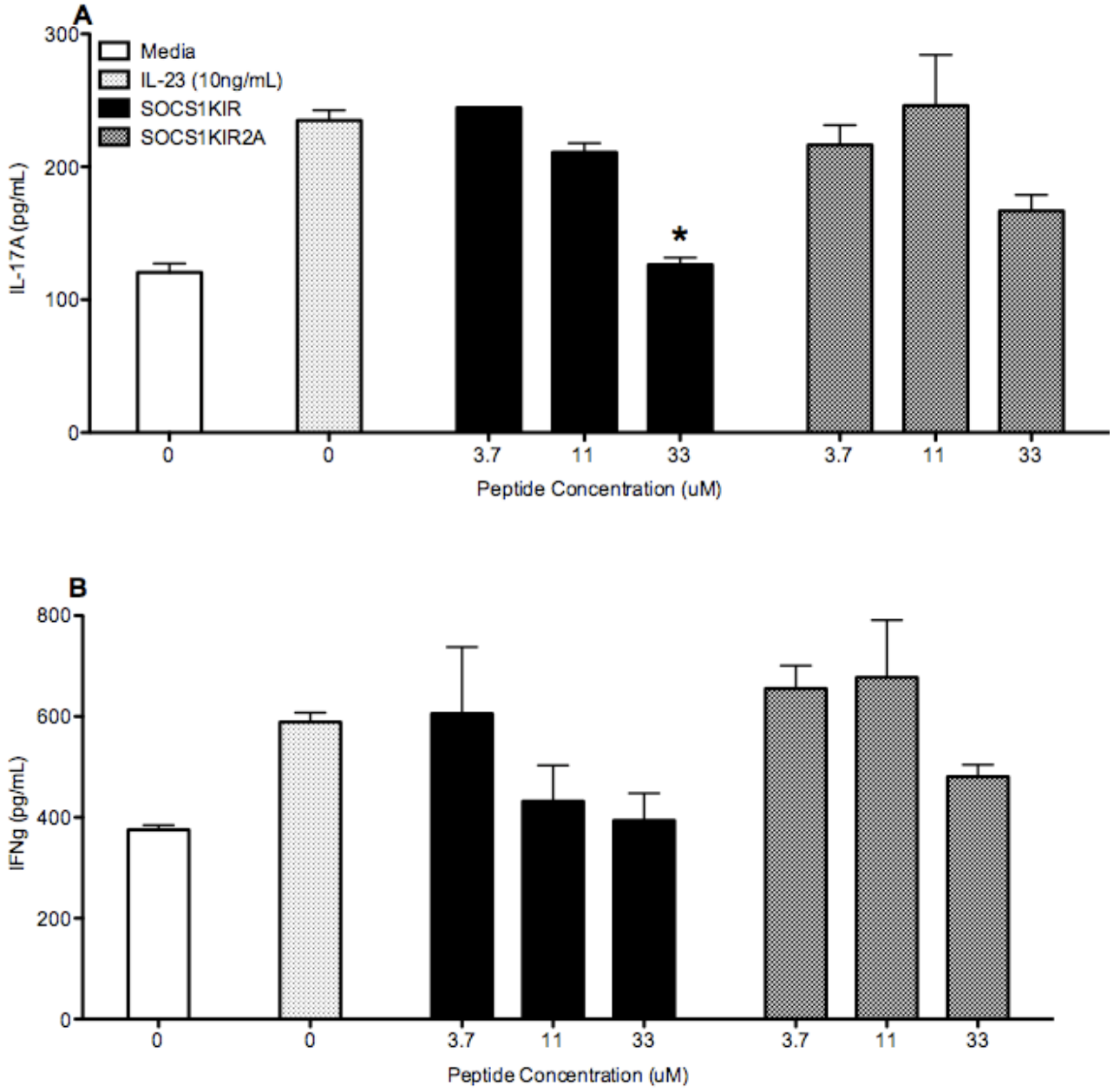
A
Media
IL-23 (10ng/mL)
SOCS1KIR
SOCS1KIR2A
IL-17A (pg/mL)
300
200
100
0
*
0
0
3.7
11
33
3.7
11
33
Peptide Concentration (uM)
B
IFNg (pg/mL)
800
600
400
200
0
0
0
3.7
11
33
3.7
11
33
Peptide Concentration (uM)

Figure 3-13. IL-23-induced induction of IL-17A and IFNγ in MBP-sensitized splenocytes is inhibited by SOCS1-KIR. A) IL-17A production by splenocytes in response to IL-23 is inhibited by SOCS1-KIR. Splenocytes were isolated from SJL/J mice 37 days after immunization with MBP. Prior to harvesting the spleen, the mouse was scored at EAE stage one. Cells were seeded at 5×10^6 cells/well in RPMI (10% FBS) in a 96-well plate. Peptides were added at the above concentrations and cells were incubated at 37°C, 5% CO_2, for 2 hours. IL-23 (10 ng/mL) was added to each well and the cells were incubated an additional 48 hours. Supernatants were collected and analyzed for IL-17A production using the IL-17A Ready-Set-Go ELISA kit (eBioscience). Absorbance was read at 490 nm. * $P < 0.01$ as determined by two-way ANOVA. B) IFNγ production by splenocytes in response to IL-23 is inhibited by SOCS1-KIR. Splenocytes were harvested and treated as described for part a. Supernatants were collected and analyzed for IFNγ production using the IFNγ Ready-Set-Go ELISA kit (eBioscience), and absorbance was read at 490 nm. For parts A and B the data are representative of four and two separate experiments, respectively.

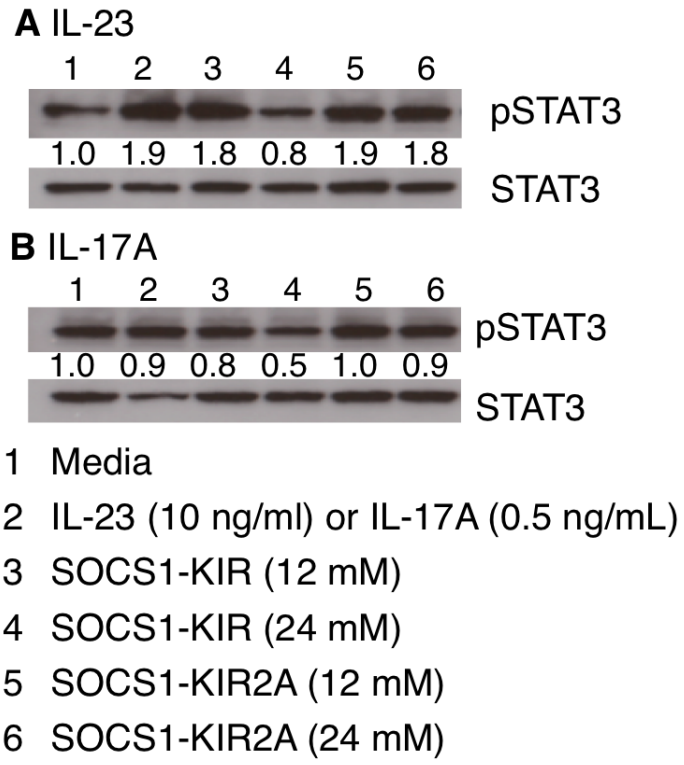

Figure 3-14. SOCS1-KIR inhibits IL-23 enhanced STAT3 phosphorylation. A) SOCS1-KIR inhibits IL-23-enhanced STAT3 phosphorylation. Splenocytes isolated from MBP-immunized mice experiencing EAE (stage 1), were treated with the above peptides for 2 hours, followed by incubation with IL-23 (10 ng/ml) for 10 minutes. The cells were lysed and protein concentration was determined by the standard BCA assay. The cell lysates were resolved using SDS-PAGE and proteins were transferred onto a nitrocellulose membrane. The membranes were probed for pSTAT3 (top) or STAT3 (bottom). Relative intensity of the bands was measured with ImageJ software (NIH) and is shown below the pSTAT3 blot. B) SOCS1-KIR inhibits STAT3 phosphorylation in splenocytes treated with IL-17. Splenocytes isolated from MBP-immunized mice were treated with the above peptides for 2 hours, followed by incubation with IL-17 (0.5 ng/ml) for 2 hours. Western blots were performed and analyzed as described for part A. Relative intensity of the bands was measured with ImageJ software (NIH). Figure courtesy of R. Dabelic.

CHAPTER 4
DISCUSSION

The immune system is composed of a wide and varied array of cells and signaling molecules that can protect the individual from diseases of both innate and foreign origin. In order to maintain proper function, the system contains within itself regulatory mechanisms to shut down the responses when the threat has been eliminated. One of these regulatory mechanisms is the induction of the suppressors of cytokine signaling, which act in a negative feedback loop to inhibit cellular responses to environmental cues that would normally stimulate an increase in inflammatory activity.

Suppressor of cytokine signaling 1 (SOCS-1) is best known for its role in inhibiting the JAK/STAT signaling cascade initiated after interferons bind to their receptors. The involvement of interferon γ (IFNγ) in the pathology of demyelinating diseases such as multiple sclerosis (MS) and its murine counterpart experimental allergic encephalomyelitis (EAE), has sparked an interest in the function of SOCS-1 in the central nervous system, but currently there is not a lot known about the expression and activity of SOCS-1 in the central nervous system (CNS) (Baker et al., 2009). Recent studies have shown that SOCS-1 is expressed in primary astrocytes after stimulation with lFNγ, that SOCS-1 and SOCS-3 are expressed in astrocytes after treatment with IFNβ, and that SOCS-1 and SOCS-3 expression in the spinal cord is increased during the onset of EAE but decreases as the disease progresses; however, it should be noted that the latter study was performed with whole spinal cord homogenates, not with isolated cells (Berard et al., 2010; Qin et al., 2008; Stark et al., 2004; Stark and Cross, 2005). Berard et al. also looked at the expression of SOCS-1 in EAE via immunohistochemistry and found that the majority of immune cells expressing SOCS-1

in the spinal cord were activated macrophages, with only a small number of T cells expressing SOCS-1 (Berard et al., 2010). The work presented here, which addressed SOCS-1 expression in isolated monocytes from the CNS of mice with active EAE, confirms that the majority of the SOCS-1 expressed by immune cells in the CNS is coming from non-$CD4^+$ monocytes, which can include microglia and macrophages, yet suggests that $CD4^+$ T cells infiltrating the CNS do not express any SOCS-1. In fact, we showed that SOCS-3 expression was moderately up-regulated in $CD4^+$ T cells and substantially increased in non-$CD4^+$ monocytes.

Previous work in our laboratory has shown that a SOCS-1 mimetic peptide, the tyrosine kinase inhibitory peptide Tkip, can inhibit the development and severity of EAE (Mujtaba et al., 2005). We have shown here that SOCS1-KIR, like Tkip, can bind to the Janus kinases JAK2 and TYK2, and inhibit the phosphorylation of STATs by these kinases in response to interferon. Elsewhere we have also shown that Tkip and SOCS1-KIR inhibited both the humoral and cellular immune response, decreasing the production of antigen-specific antibodies and blocking antigen-specific proliferation and IFNγ production by $CD4^+$ and $CD8^+$ T cells (Mujtaba et al., 2005; Waiboci et al., 2007, manuscript in preparation). Presently, however, the T helper subset being implicated in the pathology of demyelinating diseases is not T helper 1 but T helper 17. Very little is currently known about SOCS-1 and Th17 cells. Most of the research to date has focused on the role of SOCS-3 in Th17 cell development and function due to the importance of cytokines such as IL-6 and IL-23, which are known to be regulated by SOCS-3, in Th17 cell differentiation and function (Qin et al., 2009; Palmer and Restifo, 2009; Chen et al., 2006). However, Tanaka et al. showed that SOCS-1 is necessary for

the differentiation of Th17 cells by blocking the inhibitory effects of IFNγ on the activation of STAT3 and the Smads necessary for induction of the Th17 cell phenotype (Tanaka et al., 2008).

In the work presented here, we used the SOCS-1 mimetic peptide SOCS1-KIR to further our understanding of how SOCS-1 affects the course of disease in EAE mice, as well as Th17 cell function in EAE. We showed that treatment with SOCS1-KIR could reverse and inhibit monocyte infiltration into the CNS, as well as block antigen-specific proliferation of myelin basic protein (MBP) sensitized cells. We also determined that the production of IL-17A was reduced in mice treated with SOCS1-KIR and that the MBP-induced increase in IL-17A production seen in cells from the control mice was significantly limited in the SOCS1-KIR treated mice. We showed that SOCS1-KIR blocked not only the production of IL-17A in response to stimulation with MBP, but also the production of IL-17A and IFNγ in response to IL-23, which is known to be required for the development and pathogenesis of EAE (McGeachy et al., 2009). Finally, we determined that IL-23 activation of STAT3 was inhibited by treatment with SOCS1-KIR. These results indicate that a possible mechanism of action for SOCS1-KIR inhibition of Th17 cell activity might be by blocking the ability of these cells to respond to antigen presentation and IL-23, and by blocking their ability to induce the activity of other immune cells by inhibiting the production of IL-17A.

There is, however, much work that remains to be done in determining the mechanism by which SOCS1-KIR treatment exerts its therapeutic effects. For example, the above experiments do not address whether or not the reduction in IL-17A production seen in SOCS1-KIR treated mice is due to a decrease in Th17 cell activation or due to a

decrease in Th17 cell numbers. In order to address this, future studies will involve using flow cytometry to assess the percentages of Th17 cells in the spleens of treated and control mice, as well as in the splenocyte cell cultures before and after antigen stimulation. It would also be interesting to look at the percentages of Th1 cells, given the importance these cells have in EAE and in regulating Th17 cell development. Examining the production of IL-17A and IFNγ by these $CD4^+$ T cells, using intracellular staining combined with flow cytometry and ELISA of culture supernatants, would useful to assess the activity of these cells specifically.

Another aspect of SOCS1-KIR action that is not clearly delineated by the work presented is whether peptide treatment is inhibiting the recruitment of lymphocytes to the CNS or inhibiting the activation of encephalitogenic T cells that are already there. One way to address this issue is by adoptively transferring labeled MBP-specific $CD4^+$ T cells into mice with active EAE and then examining whether they migrate to the CNS, and whether this migration is inhibited in mice that have been treated with SOCS1-KIR. By using a stain such as carboxyfluorescein succinimidyl ester (CFSE), which is trapped inside the cells upon entry and gets distributed among daughter cells as they divide, it is possible to determine not only whether the adoptively transferred cells have migrated to the CNS but also whether they are proliferating there. Culturing $CD4^+$ T cells isolated from the CNS and analyzing their ability to proliferate and produce cytokines in response to antigen stimulation, and whether treatment with SOCS1-KIR affects these responses, is also a possibility; however, the number of mice with active disease required to isolate a meaningful amount of cells may be prohibitive. For example, in this

study, the brains and spinal cords of three mice were combined in order to isolate approximately 1 x 10^6 $CD4^+$ cells.

In addition to the experiments mentioned above that would enhance our understanding of the mechanism by which the SOCS1-KIR peptide alleviates EAE, much work is still needed to assess the role of SOCS-1 in EAE and multiple sclerosis in general. For example, how are the incidence and severity of experimental allergic encephalomyelitis affected in SOCS-$1^{-/-}$IFNγ$^{-/-}$ knockout mice? The loss of both the regulation provided by SOCS-1 and the inhibition of Th17 cell differentiation provided by IFNγ could lead to increased disease by allowing for greater induction and proliferation of Th17 cells, as well as increased production of their effector cytokines. At the same time, however, the role of IFNγ in allowing encephalitogenic T cells to cross the blood-brain barrier by inducing the expression of ICAM-1 and ALCAM may be required for EAE pathology (Cayrol et al., 2008; Popko et al., 1997; Kebir et al., 2009). Additionally, IL-17 has recently been shown to induce the destruction of the blood-brain barrier by stimulating the production of reactive oxygen species in brain endothelial cells and down-regulating the production of occludin which is necessary for maintaining the vascular tight junctions (Huppert 2010). Therefore, it is difficult to predict how EAE will develop in SOCS-$1^{-/-}$IFNγ$^{-/-}$ knockout mice.

Another way to study the effects of SOCS-1 on Th17 cells in mice with EAE is to decrease SOCS-1 activity without completely knocking it out. Our laboratory has developed the SOCS-1 antagonist peptide, pJAK2(1001-1013), based on the sequence of the activation loop of JAK2 (Waiboci 2007). This peptide is capable of binding to the KIR region of SOCS-1, and possesses antiviral activity against vaccinia virus, herpes

simplex virus 1, and encephalomyocarditis virus in mice (Ahmed et al., 2009; Waiboci et al., 2007; Frey et al., 2009). Because pJAK2(1001-1013) increases IFNγ signaling by inhibiting SOCS-1 activity, it is possible that treatment of mice with pJAK2 would amplify EAE due to increasing T cell infiltration of the CNS. Conversely, because IFNγ can inhibit Th17 cell development, treatment with pJAK2(1001-1013) could inhibit disease. Additionally, pJAK2(1001-1013) has been shown to increase the production of endogenous IFNβ by cells, and, as discussed above, IFNβ is capable of inhibiting the pathogenic effects of IFNγ and is the main disease-modifying agent prescribed for patients with multiple sclerosis (manuscript in preparation).

Another aspect of SOCS-1 activity in EAE that should be studied is its role in inhibiting macrophage function. Previous work by our laboratory has shown that SOCS-1 mimetic peptides can block macrophage activation, as assessed by nitric oxide production (Waiboci et al., 2007). We have also shown that one of the ways these peptides inhibit macrophage function is by blocking signaling through the toll-like receptors (TLRs), particularly those which utilize the MyD88 adaptor protein, MAL (unpublished data). TLR signaling is important in EAE, particularly in the induction phase of the disease; therefore, further elucidation of the role of SOCS-1 in TLR signaling and macrophage activation would be beneficial (Marta et al., 2009).

As mentioned above, macrophages are one of the main producers of IL-23, which is necessary for stabilizing the Th17 cell phenotype and is required for the development of EAE and lymphocyte infiltration into the CNS (Langrish et al., 2004; McGeachy and Cua, 2008; McGeachy et al., 2009; Gyulveszi et al., 2009). It would be interesting to see if SOCS1-KIR could block the production of IL-23 by macrophages as well as the

downstream signaling pathway of IL-23, as presented here. If so, this may be one of the mechanisms by which SOCS1-KIR treatment decreases the number of encephalitogenic T cells seen in this study.

Finally, the role of SOCS-1 in the differentiation and stabilization of the Th17 cell phenotype should be addressed. As discussed above, the differentiation of Th17 cells involves TGF-β, IL-6, IL-9, and/or IL-21, and the stabilization of the phenotype involves IL-23 (Elyaman et al., 2009; Wei et al., 2007; McGeachy and Cua, 2008; McGeachy et al., 2009; Chung and Dong, 2009). Because these cytokines are regulated by SOCS-3, most of the studies assessing the role of SOCS proteins in Th17 cell differentiation have looked at SOCS-3. However, SOCS-1 mimetics have previously been shown to inhibit IL-6-induced STAT3 activation (Flowers et al., 2005; Waiboci et al., 2007), and we know from the work presented herein that SOCS1-KIR inhibits IL-23 induced STAT3 activation. Therefore, one would expect that SOCS1-KIR would inhibit the differentiation of Th17 cells from naïve $CD4^+$ T cells, and that this reduction in the number of Th17 cells present in the mouse could lead to the therapeutic effects of SOCS1-KIR observed in EAE. This could be addressed by culturing naïve $CD4^+$ T cells in the presence of the above cytokines, with and without SOCS1-KIR, and monitoring the differentiation of the cells in to Th17 cells via flow cytometry or ELISA for IL-17A production.

As can be seen by the above discussions, the pathology of demyelinating diseases such as multiple sclerosis and experimental allergic encephalomyelitis will not likely be ascribed to a single cell type or cytokine. Subsequently, developing therapies for these diseases is equally complex. Therefore, continual research into the roles of

Th1 and Th17 cells, their respective cytokines, and their regulators such as SOCS-1 is necessary to further our understanding of these and other autoimmune disorders.

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BIOGRAPHICAL SKETCH

Lindsey Delores Jager was born in Spicer, Minnesota, in 1979. After completing high school, she attended The Colorado College, Colorado Springs, CO, graduating in 2002 with a Bachelor of Arts in anthropology. Two years of completing the basic science requirements for applying to medical school, one at Linfield College, Portland OR, and one at the University of Wisconsin, Madison, WI, instilled her with a desire to focus on medical research in lieu of medical practice. In 2006, she graduated from the College of Health Sciences at Midwestern University, Downers Grove, IL, with a Master of Biomedical Science. That August, she arrived in Gainesville, FL, to begin her journey of doctoral research in the Department of Microbiology and Cell Science at the University of Florida.