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The Kinase Inhibitory Region of SOCS-1 is Sufficient to Inhibit T-helper 17 and other Immune Functions in Experimental Allergic Encephalomyelitis

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Abstract

Suppressors of cytokine signaling (SOCS) negatively regulate the immune response, primarily by interfering with the JAK/STAT pathway. We have developed a small peptide corresponding to the kinase inhibitory region (KIR) sequence of SOCS-1, SOCS1-KIR, which inhibits kinase activity by binding to the activation loop of tyrosine kinases such as JAK2 and TYK2. Treatment of SJL/J mice with SOCS1-KIR beginning 12 days post-immunization with myelin basic protein (MBP) resulted in minimal symptoms of EAE, while most control treated mice developed paraplegia. SOCS1-KIR treatment suppressed interleukin-17A (IL-17A) production by MBP-specific lymphocytes, as well as MBP-induced lymphocyte proliferation. When treated with IL-23, a key cytokine in the terminal differentiation of IL-17-producing cells, MBP-sensitized cells produced IL-17A and IFN γ ; SOCS1-KIR was able to inhibit the production of these cytokines. SOCS1-KIR also blocked IL-23 and IL-17A activation of STAT3. There is a deficiency of SOCS-1 and SOCS-3 mRNA expression in CD4⁺ T cells that infiltrate the CNS, reflecting a deficiency in regulation. Consistent with therapeutic efficacy, SOCS1-KIR reversed the cellular infiltration of the CNS that is associated with EAE. We have shown here that a SOCS-1 like effect can be obtained with a small functional region of the SOCS-1 protein that is easily produced.

Keywords

suppressors of cytokine signaling; experimental allergic encephalomyelitis; multiple sclerosis; mimetic peptide

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1. Introduction

The immune system is remarkably complex and flexible, comprised of innate and adaptive arms that allow rapid responses to a variety of threats to the individual. This system contains within itself regulators such as regulatory lymphocytes and suppressors of cytokine signaling (SOCS). These regulators are required in order to govern and limit the extent of the response of the effector arm of the immune system. An unregulated immune system quickly turns on the host and becomes a problem rather than a solution to threats on the individual.

Multiple sclerosis (MS) is a T-cell-mediated autoimmune disease that targets the myelin sheath of neurons of the central nervous system (CNS) (Lassmann et al., 2007; Stuve, 2009). It is a well-known example of dysregulation of the immune system where, for reasons not fully understood, the regulatory arms of the immune system fail in 250,000 to 400,000 Americans (Lassmann et al., 2007; Stuve, 2009). It was widely felt that T-helper 1 (Th1) cells driven by the cytokine interleukin-12 (IL-12) were primarily responsible for the CNS pathology of MS with gamma interferon (IFN γ) as the effector cytokine (El Behi et al., 2010). More recently, IL-17 producing CD4⁺ T cells (Th17 cells) have supplanted Th1 cells as the primary cause of MS (El Behi et al., 2005; El Behi et al., 2010; Korn et al., 2009; Boniface et al., 2008; Linker et al., 2009).

Experimental allergic encephalomyelitis (EAE) is a widely studied model of MS, with a view toward understanding the mechanism of disease, as well as therapeutic approaches to treatment (El Behi et al., 2005; Ercolini et al. 2006). It is induced in mice, rats, and primates by immunization with proteins or peptides of the myelin sheath. We are particularly interested in manipulation of the SOCS arm of immune regulation as a therapeutic approach to the treatment of MS and EAE. SOCS are a family of eight proteins of which two, SOCS-1 and SOCS-3, are of interest to the natural regulation of the immune system with respect to MS and EAE and as targets of approaches to treating these diseases. SOCS-1 and SOCS-3 are structurally similar with, starting N-terminally, a 12-amino acid kinase inhibitory region (KIR), a large SH2 domain, and a 40-amino acid C-terminal SOCS box that is involved in proteasomal degradation of SOCS and its associated tyrosine kinases (Yasukawa et al., 1999; Alexander and Hilton, 2004; Yoshimura et al., 2007; Dalpke et al., 2008; Croker et al., 2008; Babon et al., 2009). KIR and SH2 are involved in binding to tyrosine kinases with inhibition of catalytic activity (Babon et al., 2009; Croker et al., 2008; Waiboci et al., 2007).

We have been interested in the interaction of SOCS-1 with the activation loop of the JAK2 tyrosine kinase. Accordingly, we designed a short 12-mer peptide, WLVEFFVIFYFRR, which binds to the activation loop of JAK2, resulting in inhibition of its autophosphorylation, as well as its phosphorylation of the IFN γ receptor subunit IFNGR-1 (Flowers et al., 2004; Waiboci et al., 2007). This tyrosine kinase inhibitory peptide (Tkip) was developed based on hydrophobic complementarity to the activation loop of JAK2 as per the peptide pJAK2(1001–1013) with the tyrosine at position 1007 being phosphorylated (Flowers et al., 2004). Tkip did not bind to nor inhibit tyrosine phosphorylation of vascular endothelial growth factor receptor or phosphorylation of a substitute peptide by the proto-oncogene tyrosine kinase c-Src. Tkip, as with SOCS-1, inhibited EGF receptor autophosphorylation. It has been suggested that SOCS-1 specifically recognizes the autophosphorylation sequence 1001–1013 containing the phosphotyrosine residue (pY1007) in the activation loop of JAK2 and that phosphorylation of Y1007 is required for activation (Yasukawa et al., 1999; Flowers et al., 2004; Waiboci et al., 2007). SOCS-1 binding blocks JAK2-mediated tyrosine phosphorylation of its substrate. Tkip recognizes both unphosphorylated and phosphorylated Y1007, though it has a higher affinity for pY1007. Thus, Tkip recognizes the JAK2 autophosphorylation site similar to SOCS-1. Tkip has also

been shown to inhibit proliferation of prostate cancer cells, and to block JAK2-mediated phosphorylation and activation of the oncogene STAT3 (Flowers et al., 2005). Furthermore, Tkip has been shown to protect mice from EAE via blocking JAK2 activation by inflammatory cytokines (Mujtaba et al., 2005).

We recently showed that a peptide corresponding to the KIR of SOCS-1, ⁵³DTHFRTFRSHSDYRRI (SOCS1-KIR), bound to the autophosphorylation site of JAK2, pJAK2(1001–1013) (Waiboci et al., 2007). Cells treated with palmitated SOCS1-KIR for plasma membrane penetration inhibited IFN γ -induced STAT1 α phosphorylation, inhibited IFN γ activation of RAW264.7 macrophages, and inhibited antigen-specific splenocyte proliferation. These results suggested that SOCS1-KIR, like Tkip, could function as a SOCS-1 mimetic.

In this study, we have evaluated the therapeutic effects of SOCS1-KIR in a mouse model of relapsing/remitting EAE. Treatment of myelin basic protein (MBP)-immunized mice with SOCS1-KIR inhibited severe relapsing paralysis in these mice. Further, the protection of mice against EAE was associated with inhibition of the Th17 anti-MBP response in the mice. Recent studies suggest that SOCS-3 is the key SOCS molecule in regulation of EAE via inhibition of Th17 cells, while SOCS-1 is suggested to enhance EAE by inhibiting the suppressive effects that the Th1 cytokine IFN γ has on Th17 cells (Tanaka et al., 2008). Our results here with the SOCS-1 mimetic SOCS1-KIR would suggest that SOCS-1, when acting on Th17 cells, has an inhibitory effect on Th17-induced EAE in SJL/J mice.

2. Materials and Methods

2.1 Peptide synthesis

Peptides were synthesized using conventional fluorenylmethyloxycarbonyl chemistry, as previously described (Szenté et al., 1994), on 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 high-performance liquid chromatography (HPLC). They were then dissolved in DMSO or PBS prior to use (Sigma-Aldrich, St Louis, MO). The peptides used in this study are presented in Table 1.

2.2 Mice

The Institutional Animal Care and Use Committee at the University of Florida approved all of the animal protocols mentioned herein. Female SJL/J mice (6 to 8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in standard SPF facilities.

2.3 Induction of EAE, evaluation of clinical disease, and administration of peptides

On day 1, SJL/J mice were injected with 300 μ g/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 and 400 ng/mouse pertussis toxin (List Biological Laboratories Inc, Campbell, CA) in PBS i.p. On day 3, the pertussis toxin injection was repeated (Mujtaba et al., 2005). Beginning on day 12 post-immunization, after lymphocyte infiltration of the CNS had begun, mice were administered the following treatments or peptides every other day via i.p. injection in 100 μ l final volume: PBS, SOCS1-KIR (60 μ g/mouse), or SOCS1-KIR 2A (60 μ g/mouse). The mice were monitored daily for signs of EAE and graded according to

the following scale: 0, normal; 1, loss of tail tone; 2, hind limb weakness; 3, paraparesis; 4, paraplegia; 5, moribund; and 6, death.

2.4 Detection of IL-17A and IFN γ production

SJL/J mice were immunized with MBP for EAE induction as described above and had been receiving i.p. injections of 100 μ l PBS, SOCS1-KIR (60 μ g/mouse), or SOCS1-KIR2A (60 μ g/mouse) every other day beginning day 12 post-immunization. Spleens were harvested at the indicated times post-immunization when the mice were scored at EAE Stage 1. Splenocytes were seeded at 5×10^6 cells/well in RPMI (10% FBS). For detection of basal levels of IL-17A, splenocytes were incubated in RPMI (10% FBS) for 24 hours at 37°C, 5% CO₂. For IL-17A production in response to MBP stimulation, splenocytes were treated with or without 25 μ g/ml MBP and incubated for 24 hours. Supernatants were collected and analyzed for IL-17A by ELISA using the IL-17A Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).

In order to determine if SOCS1-KIR can inhibit IL-17A production in response to MBP, splenocytes were isolated from MBP-immunized mice treated with PBS as described above. Peptides were added at 0, 3.7, 11, and 33 μ M concentrations and cells were incubated at 37°C, 5% CO₂ for 2 hours. MBP was then added to each well at 50 μ g/ml and the cells were incubated an additional 24 hours. Supernatants were collected and analyzed for IL-17A by ELISA.

In order to determine if SOCS1-KIR can inhibit IL-17A and IFN γ production in response to IL-23, splenocytes from MBP-immunized mice treated with PBS as described above. Peptides were added at 0, 3.7, 11, and 33 μ M concentrations and cells were incubated at 37°C, 5% CO₂ for 2 hours before the addition of IL-23 (10 ng/ml). Splenocytes were then incubated an additional 48 hours. Supernatants were collected and analyzed for IL-17A as above or IFN γ using the IFN γ Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).

2.5 Splenocyte proliferation assay

Spleens were harvested from MBP-immunized SJL/J mice at EAE stage 1. Splenocytes were isolated and seeded at 5×10^6 cells/well in RPMI (10% FBS) in a 96-well plate. Peptides were added at 0, 3.7, and 11 μ M concentrations and cells were incubated at 37°C, 5% CO₂ for 2 hours. MBP (50 μ g/ml) 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).

2.6 Inhibition of IL-23 and IL-17A induced STAT3 activation

Splenocytes isolated from MBP-immunized SJL/J mice experiencing EAE stage 1, were treated with SOCS1-KIR or SOCS1-KIR2A at 12 and 24 μ M for 2 hours, followed by incubation with IL-23 (10 ng/ml) (eBioscience, San Diego, CA) for 10 minutes or IL-17A (100 ng/ml) (R&D Systems, Minneapolis, MN) for 2 hours at 37°C, 5% CO₂. 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 bicinchoninic acid 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).

2.7 Histology

For analysis of CNS infiltration, animals were sacrificed as described above on days 12 or 38 post-immunization with MBP and their brains were collected in 4% paraformaldehyde

(PFA) in PBS and fixed overnight. They were then stored in 70% ethanol until processing. 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). Cut sections were stained with hematoxylin and eosin using SelectTech 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). To visualize SOCS1-KIR in the CNS, SOCS1-KIR was conjugated to FITC using the EZ-Label FITC Protein Labeling Kit (Pierce, Rockford, IL). Naïve and MBP-sensitized mice at EAE stage 1 were injected with 1 ml FITC-SOCS1-KIR (50 µg/ml) or an equivalent amount of FITC alone and sacrificed 2 hours later. Brains were collected and processed as above, without H&E staining, and sections were dried in the dark for 24 hours before analysis.

2.8 Quantitative Real-Time PCR (qRT-PCR)

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). 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 1×10^6 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'-TGGGACAGAGGGCATTAAAG-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 s, 60°C for 5 s, and 72°C for 15 s. 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.

2.9 Inhibition of IFN γ production by CD4⁺ and CD8⁺ T cells

Splenocytes were isolated as described from mice with active EAE then enriched for the desired cell type using Dynal Biotech negative isolation kits (Invitrogen, Carlsbad, CA). CD4⁺ and CD8⁺ cell suspensions were determined to be > 90% pure by FACS analysis. Cells were then seeded at 1×10^5 cells/well and incubated with media, MBP (50 µg/ml), paraformaldehyde-fixed antigen presenting cells, and SOCS1-KIR or the control peptide for 48 hours, 37°C, 5% CO₂. Cells were then transferred to IFN γ -ELISPOT plates for 48 hours, after which they were washed, incubated with secondary antibody for 1 hour, washed, and incubated with substrate and allowed to develop according to the manufacturer's instructions (Mabtech Inc., Mariemont, OH). The plates were then blotted dry and spots indicating IFN γ -producing cells were counted.

2.10 Macrophage activation assay

Murine macrophages (RAW264.7) were seeded on 24-well plates at a concentration of 3×10^5 cells/well and allowed to adhere. Varying concentrations of peptides were then added to the cells and incubated for 2 hours at 37°C, 5% CO₂. Purified lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) at various concentrations was then added and incubated for an additional 48 hours at 37°C. Supernatants were transferred into fresh tubes and assayed for nitrite levels as a measure of nitric oxide production using Griess reagent according to the manufacturer's instructions (Alexis Biochemicals, Plymouth Meeting, PA).

2.11 Binding assays

Binding assays were performed as previously described (Flowers et al. 2004). Peptides were bound to 96-well plates in binding buffer (0.1 M sodium carbonate-sodium bicarbonate, pH 9.6). The wells were washed three times with wash buffer (0.9% NaCl and 0.05% Tween-20) and incubated in blocking buffer (2% gelatin and 0.05% Tween-20 in PBS) for 1 hour at room temperature. Then, the wells were washed three times with wash buffer and incubated with various concentrations of biotinylated peptides for 1 hour at room temperature. The wells were washed five times with wash buffer and bound biotinylated peptides were detected using HRP-conjugated neutravidin (Invitrogen, Carlsbad, CA) and *o*-phenylenediamine in stable peroxidase buffer (Pierce Biochemicals, Rockford, IL). The chromogenic reaction was stopped by the addition of 2 M H₂SO₄ (50 µl) to each well. Absorbance was measured at 490 nm using a microplate reader (Bio-Tek, Winooski, VT).

2.12 Western blot analysis of MAL degradation

RAW264.7 cells were incubated with peptides for 2 hours, and then with LPS (1 µg/ml) for 30 or 60 minutes. The cells were washed in cold PBS and harvested in RIPA buffer containing protease and phosphatase inhibitor cocktails (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Protein concentration was measured using a BCA kit (Pierce Biochemicals, Rockford, IL) and lysates were resolved with SDS-PAGE, transferred onto nitrocellulose membranes, and probed with anti-MAL antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Detection of proteins was accomplished using ECL Protein Detection Reagents (Amersham Biosciences, Piscataway, NJ).

3. Results

3.1 Therapeutic efficacy of SOCS1-KIR in treatment of mice with severe relapsing/remitting EAE

SJL/J mice were immunized with bovine MBP for development of relapsing/remitting EAE as previously described (Mujtaba et al., 2005). Initiation of early stage EAE was confirmed histologically by lymphocyte infiltration of the CNS by day 12. Starting on day 12 postimmunization, mice were injected i.p. every-other day with PBS (control), 60 µg SOCS1-KIR, or 60 µg SOCS1-KIR2A (alanine-substituted for phenylalanine at residues 56 and 59; see Table 1). The phenylalanines were previously determined to be critical for KIR function (Yasukawa et al., 1999). Severe EAE developed in the control mice at 47 to 62 days with an average disease score of greater than 4 (paraplegia) at 60 days (Figure 1). In contrast, SOCS1-KIR treated mice had an average disease score of less than 2. SOCS1-KIR2A treated mice had disease severity intermediate between the control and SOCS1-KIR treated mice. SOCS1-KIR2A had previously been shown to have reduced inhibitory effect on STAT1α activation in cells treated with the type I interferon IFNτ compared to SOCS1-KIR (Ahmed et al., 2009). Thus, SOCS1-KIR therapeutically protected mice against severe relapsing/remitting EAE.

3.2 IL-17 production is inhibited in splenocytes of MBP immunized mice treated with SOCS1-KIR

T cells of the IL-17-producing phenotype (Th17) have been identified as one of the central effector cells in the pathology of EAE and MS (reviewed in Boniface et al., 2008; Korn et al., 2009; McGeachy et al., 2009; El Behi et al., 2010:). Accordingly, splenocytes from SJL/J mice as in Figure 1 were isolated 36 days after MBP injection and production of IL-17A was measured after 24 hours in culture. Mice treated with PBS produced approximately 60 pg/ml of IL-17A, while splenocytes from mice treated with SOCS1-KIR produced less than 10 pg/ml of IL-17A, as shown in Figure 2A. Consistent with disease intermediate between PBS and SOCS1-KIR treatment, SOCS1-KIR2A treated mice produced approximately 22 pg/ml IL-17A. Thus, SOCS1-KIR treated mice produced at least 6-fold less IL-17A, indicating that SOCS1-KIR had an inhibitory effect on the Th17 cell phenotype.

IL-17A production was also measured after treatment of splenocytes in culture with MBP. Splenocytes from PBS, SOCS1-KIR, and SOCS1-KIR2A treated mice were isolated 27 days post-immunization with MBP and treated for 24 hours with 25 µg/ml of MBP before IL-17A levels in the culture supernatants were quantified. As shown in Figure 2B, MBP induced approximately 400 pg/ml of IL-17A by splenocytes from MBP immunized mice that were treated with PBS. By contrast, the same concentration of MBP induced approximately 8-fold less IL-17A by splenocytes from SOCS1-KIR treated mice. Splenocytes from SOCS1-KIR2A treated mice had reduced IL-17A production, approximately 4-fold less than that produced by splenocytes from PBS treated mice. Thus, the inhibitory effect of SOCS1-KIR on IL-17A producing cells was observed even when the cells were stimulated with MBP in culture.

We were also interested in whether SOCS1-KIR could inhibit the production of IL-17A by T cells known to be sensitized to MBP. Accordingly, splenocytes were isolated from SJL/J mice 36 days after immunization with MBP. The mice had active EAE at the time of isolation of the cells. The cells were stimulated with 50 µg/ml MBP 2 hours after peptide treatment and IL-17A levels were determined 24 hours later. SOCS1-KIR inhibited IL-17A production by approximately 2.5-fold at 33 µM, as shown in Figure 2C, while SOCS1-KIR2A inhibited at approximately 1.4-fold. SOCS1-KIR can thus inhibit the induction and/or function of T-helper cells. Further, SOCS1-KIR has recently been shown to block T helper 17 cell polarization by IL-6 and TGFβ (Joseph Larkin III, manuscript in preparation). This is consistent with the therapeutic efficacy of SOCS1-KIR in mice with active EAE pathology.

3.3 SOCS1-KIR inhibits MBP-induced proliferation of splenocytes from EAE mice

We were interested in determining the ability of SOCS1-KIR to inhibit antigen-specific proliferation of splenocytes in addition to inhibiting IL-17A production. This was assessed using splenocytes from MBP-immunized SJL/J mice that had active EAE disease. As shown in Figure 3, treatment of splenocytes with 11 µM SOCS1-KIR significantly inhibited MBP-specific proliferation while SOCS1-KIR2A was not inhibitory. Similar to inhibition of IL-17A production, SOCS1-KIR inhibited MBP-specific proliferation of splenocytes, which is consistent with its therapeutic efficacy in ongoing EAE.

3.4 SOCS1-KIR prevents cellular infiltration into the CNS of MBP-immunized mice

SOCS1-KIR treatment of MBP-immunized mice was initiated 12 days after MBP injection. Figure 4A (see inset) shows that cellular infiltration of the central nervous system (CNS) has already begun at that time, compared to non-immunized mice (Figure 4B), particularly near the vessels lining the ventricular spaces. We were interested in determining if SOCS1-KIR treated mice were protected from cellular infiltration of the CNS at day 38 post-

immunization with MBP as compared to untreated mice. As shown in Figure 4C, PBS-treated mice had extensive cellular infiltration (see inset), while the SOCS1-KIR treated mice had no cellular infiltration in the brain (Figure 4D). SOCS1-KIR2A, with reduced protective ability, also had cellular infiltration of the brain (Figure 4E), similar to the PBS-treated mice. Thus, the therapeutic treatment with SOCS1-KIR protected the mice from cellular infiltration of the brain. Importantly, the SOCS1-KIR therapy also results in a reversal of cellular infiltration that had occurred prior to treatment.

3.5 SOCS1-KIR enters the endothelial cells of the blood brain barrier (BBB)

Given that SOCS1-KIR inhibits infiltration of lymphocytes into the CNS, we were interested in determining if the peptide was present in the CNS after i.p. injection of EAE mice. Accordingly, we coupled SOCS1-KIR to FITC, FITC-SOCS1-KIR, and monitored its appearance in the brain two hours post-injection. As shown in Figure 5A, FITC-SOCS1-KIR was taken up by endothelial cells in the blood vessels of the meninges of EAE mice, while the control of FITC alone was not detected (Figure 5B). Neither FITC-SOCS1-KIR (Figure 5C) nor FITC alone (Figure 5D) were detected in the BBB endothelial cells of naïve mice. Thus, SOCS1-KIR entered the BBB endothelial cells of EAE mice, but not naïve mice. This suggests that SOCS1-KIR enters CNS endothelial cells that have been compromised as a result of the immunological events associated with EAE (Abbott et al., 2010). The absence of infiltrating lymphocytes in the CNS of SOCS1-KIR treated mice would suggest that it restores the functional integrity of the BBB of these mice.

3.6 CNS infiltrating CD4⁺ T cells do not express SOCS-1 mRNA and express SOCS-3 mRNA at relatively low levels

Given the therapeutic efficacy of SOCS1-KIR, we were interested in the expression of endogenous SOCS-1 and SOCS-3 mRNA in CD4⁺ T cells and non-CD4⁺ monocytes during active EAE. Accordingly, we performed quantitative real-time PCR (qRT-PCR) to determine SOCS-1 and SOCS-3 mRNA expression in CNS infiltrating CD4⁺ T cells, as well as splenic CD4⁺ T cells. Expression of both SOCS mRNAs in splenic cells from EAE mice increased slightly above that of naïve CD4⁺ T cells from the spleen, while in the CNS CD4⁺ T cells did not express SOCS-1, and SOCS-3 mRNA increased approximately 6-fold over that of the naïve splenic CD4⁺ T cells (Figure 6). CD4⁺ T cells were not present in the CNS of naïve mice. Non-CD4⁺ splenic monocytes expressed low levels of SOCS-1 and SOCS-3 mRNA in both EAE and naïve mice. In the CNS, non-CD4⁺ monocytes also expressed low levels of SOCS-1 mRNA. The striking result was the relatively high levels of SOCS-3 mRNA in these cells, which was approximately 60-fold greater than in naïve cells.

Thus, the expression of SOCS-1 mRNA in CNS infiltrating CD4⁺ T cells was absent in EAE mice and low in the spleen. SOCS-3 mRNA expression in the CNS infiltrating CD4⁺ T cells was higher, approximately 6-fold, than in the spleen, but was approximately 60-fold higher in the non-CD4⁺ monocytic cells of the CNS of EAE mice. The dominant SOCS mRNA expression in the CNS of EAE mice appears to be of SOCS-3 and this occurs primarily in the non-CD4⁺ monocytes. These observations may be a factor in the effector phase of EAE in mice where SOCS1-KIR treatment is effective as a therapeutic.

3.7 SOCS1-KIR inhibits IL-23 induction of IL-17A and IFN γ in splenocytes of MBP-immunized mice

IL-23 is required for the terminal differentiation of IL-17 cells and the associated induction of IL-17 (McGeachy et al., 2009). To determine the effect of SOCS1-KIR on IL-17A production by cells treated with IL-23, splenocytes were isolated from SJL/J mice with MBP-induced EAE 37 days post-immunization. The cells were incubated with IL-23, 10 ng/ml, in the presence or absence of SOCS1-KIR for 48 hours. In the absence of SOCS1-KIR,

IL-23-treated cells increased IL-17A production about two-fold over that of the control. SOCS1-KIR, 33 μ M, blocked the induction to the background level, while SOCS1-KIR2A was less inhibitory (Figure 7A). Thus, SOCS1-KIR inhibited the effector function of IL-23, which is consistent with its protection of MBP-sensitized mice from on-going, progressively severe EAE.

Recent studies have shown that a subset of Th17 cells from MS patients and mice with EAE produce IFN γ in addition to IL-17A when stimulated with IL-23 (Kebir et al., 2009; Abromson-Leeman et al., 2009). Further, IFN γ -producing Th17 cells preferentially crossed the blood-brain barrier in MS and accumulated in the CNS of mice during the effector phase of EAE (Kebir et al., 2009). Thus, we determined IFN γ levels in the IL-23 treated cultures and the effect of SOCS1-KIR on IFN γ production. As shown in Figure 7B, IL-23 significantly increased IFN γ production in treated cells, which was significantly suppressed by 11 μ M SOCS1-KIR. SOCS1-KIR2A, as in IL-17A production, was much less effective at inhibiting the induction of IFN γ by IL-23. Thus, SOCS1-KIR inhibited both IL-17A and IFN γ production in cells treated with IL-23. Given that IFN γ ⁺ Th17 lymphocytes may be important for the CNS damage of MS and EAE, the blocking of their infiltration into the CNS by SOCS1-KIR is likely key in its protection against EAE.

3.8 SOCS1-KIR inhibits MBP-induced IFN γ production by CD4⁺ and CD8⁺ T cells

Because of the important role IFN γ plays in EAE, we were interested in determining the effect of SOCS1-KIR on T cells sensitized to MBP. To do this, we isolated CD4⁺ and CD8⁺ T cells from the spleens of MBP-immunized SJL/J mice exhibiting signs of active EAE. The cells were incubated with the peptides in the presence of inactivated APCs and MBP. Following transfer to IFN γ -ELISPOT plates, the cells were incubated for 48 hours, and spots representing IFN γ -producing cells were counted. As can be seen in Figure 8A, SOCS1-KIR inhibited MBP-induced IFN γ production by CD4⁺ T cells at 11 μ M, while the control peptide did not. Similarly, IFN γ production by CD8⁺ T cells was inhibited by SOCS1-KIR. In this case, as shown in Figure 8B, a dose as low as 1.2 μ M was sufficient to block IFN γ production. Therefore, in conjunction with the data presented above, SOCS1-KIR inhibits antigen-induced and IL-23 stimulated production of IFN γ in mice with active EAE.

3.9 SOCS1-KIR inhibits IL-23 and IL-17A enhanced STAT3 activation

As a functional correlate at the level of signal transduction, we determined 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 9A, cells from these mice had constitutive pSTAT3, probably as a result of ongoing disease, which was enhanced approximately 2-fold by treatment with IL-23 (10 ng/ml). SOCS1-KIR at 24 μ M inhibited IL-23 activation of STAT3 to the level of that seen in untreated cells. SOCS1-KIR2A had no effect on IL-23 activation of STAT3. Thus, SOCS1-KIR inhibits IL-23 function at the level of signal transduction.

Splenocytes from EAE mice were also treated with IL-17A (100 ng/ml). SOCS1-KIR, at 12 and 24 μ M, reduced the level of pSTAT3 below that seen constitutively (Figure 9B). As with many of the above experiments, SOCS1-KIR2A had a reduced inhibitory effect compared to SOCS1-KIR. Thus, SOCS1-KIR has an inhibitory effect on the effector cytokines IL-23 and IL-17A in ongoing EAE at the level of signal transduction.

3.10 SOCS1-KIR regulates Toll-like receptor (TLR) signaling

Although the precise role of TLR signaling in EAE is not yet known, TLRs such as TLR2 promote progressive EAE (Farez et al., 2009; Marta et al., 2009). We were therefore interested in determining whether SOCS1-KIR would be able to regulate TLR signaling.

MyD88 is a central adapter protein shared by most TLRs. TLR2 and TLR4 signaling via MyD88 involves activation of the adapter protein MAL (TIRAP) by Bruton's tyrosine kinase (Btk), resulting in MAL-dependent p65 phosphorylation and the resultant activation of NF- κ B (Fitzgerald et al., 2001). Induced SOCS-1 modulates this activation by binding to activated MAL, causing ubiquitination, followed by proteasomal degradation of MAL (Mansell et al., 2006). In order to determine the effect of SOCS1-KIR on TLR4 signaling, we treated murine RAW264.7 macrophages with SOCS1-KIR. As shown in Figure 10A, SOCS1-KIR inhibited LPS-induced nitric oxide (NO) production. Based on a similar hydropathic profile (Kyte and Doolittle, 1982) of a candidate tyrosine kinase phosphorylation site on MAL to the activation loop of JAK2, JAK2(1001–1013) (Figure 10B), we synthesized a biotinylated MAL(82–94) peptide (see Table 1) and determined its ability to bind to SOCS1-KIR. As shown in Figure 10C, MAL(82–94) showed a similar binding pattern to SOCS1-KIR as that of JAK2(1001–1013). Another tyrosine kinase phosphorylation site on MAL, MAL(154–166), which had a hydropathic profile different from JAK2(1001–1013), did not bind to SOCS1-KIR.

We hypothesized that the binding of MAL(82–94) to SOCS1-KIR reflected the mechanism of SOCS-1 recognition of MAL in LPS-treated cells. Accordingly, we treated RAW264.7 macrophages with SOCS1-KIR and MAL(82–94) to compete, respectively, with SOCS-1 for binding to MAL and with MAL for binding to SOCS-1. As shown in Figure 10D, LPS treatment of cells in the presence of control peptide showed essentially complete degradation of MAL by 60 minutes, while LPS treatment in the presence of SOCS1-KIR completely blocked MAL degradation. Similarly, MAL(82–94) treatment also protected against MAL degradation. The fact that SOCS1-KIR inhibits LPS activation of macrophages by binding to a critical functional site on MAL, yet blocks SOCS-1 mediated degradation of MAL, suggests that inhibition of function can occur through binding, without the necessity for MAL degradation.

4. Discussion

There is not much known concerning SOCS and the CNS in autoimmune neurological disorders. Interest resides in the expression and function of SOCS-1 and SOCS-3 in the cells and tissues of the CNS, as well as in the infiltrating immune cells. Treatment of primary astrocytes with IFN β induced the expression of both SOCS-1 and SOCS-3 at the transcriptional and protein levels (Qin et al., 2008). It was shown that SOCS-1 expression occurred through activated STAT1 α , while SOCS-3 was induced by activated STAT3. The induction of SOCS-1 and SOCS-3 in astrocytes inhibited the production of chemokines that play a role in lymphocyte infiltration into the CNS. It was concluded that IFN β induction of SOCS-1 and SOCS-3 functioned to attenuate chemokine-related inflammation in the CNS. IFN β is a therapeutic for MS (Javed and Reder, 2006; Stuve et al., 2009), and its induction of SOCS in astrocytes and other non-lymphoid cells in the CNS could play a role in its therapeutic efficacy.

It has been reported that the loss of SOCS-1 in T cells results in the dysregulation of Th1 and IFN γ activity, which suppress Th17 function, resulting in less severe EAE (Tanaka et al., 2008). In our model, it seems that the absence of SOCS-1 in CNS infiltrating T cells is not associated with protection from EAE. The qRT-PCR results for SOCS-1 and SOCS-3 mRNA in infiltrating CD4⁺ T cells in the CNS of mice with active EAE showed an absence of SOCS-1 mRNA and a relatively modest increase in SOCS-3 mRNA. Splenic CD4⁺ T cells from the same mice showed modest levels of both SOCS-1 and SOCS-3 mRNA, similar to that of cells from naïve mice. One striking result was the relatively high levels of SOCS-3 mRNA in non-CD4⁺ monocytic cells in the CNS compared to the low levels of SOCS-1 mRNA. These SOCS-3 expressing cells are most likely of the macrophage,

microglia, or dendritic cell lineage. Effector CD4⁺ T cells that have infiltrated the CNS are most likely at the stage where they can act on CNS targets without the requirement of accessory cells (Oukka, 2007). Thus, the SOCS arm of the regulatory brakes on these cells is missing or significantly reduced. The therapeutic efficacy of SOCS1-KIR, then, is most likely to augment this reduced endogenous SOCS function.

We have previously developed a novel tyrosine kinase inhibitory peptide, Tkip, which is a mimetic of SOCS-1. Tkip inhibits JAK2 phosphorylation of STAT1 α (Waiboci et al., 2007; Flowers et al., 2004; Ahmed et al., 2009). We showed that Tkip therapeutically protected SJL/J mice against relapsing/remitting EAE induced by MBP. The severity of disease in these mice was significantly lower than that reported here. Nonetheless, both the humoral and cellular arms of the anti-MBP response were suppressed. The role of Th17 effector cells and cytokines, however, was not ascertained with Tkip. Tkip does not represent any sequence in SOCS-1, but we showed that it functioned similarly to the kinase inhibitory region (KIR) of SOCS-1. This led to the characterization of the KIR in terms of its interaction with JAK2.

Our observation that SOCS1-KIR binds to the activation loop of JAK2, as per pJAK2(1001–1013), was antithetical to the prevailing view that it functioned as a pseudosubstrate and bound to the autocatalytic site of JAK2 (Waiboci et al., 2007; Ahmed et al., 2009). SOCS-1 protein competed with SOCS1-KIR in binding to pJAK2(1001–1013), suggesting that the two recognized the JAK2 activation loop in a similar fashion (Waiboci et al., 2007). We also showed that SOCS1-KIR bound to the activation loop of TYK2. The activation loops of both kinases share a high sequence homology and structural similarity, which is reflected by similar hydropathic profiles (Ahmed et al., 2009). This would suggest that SOCS1-KIR should inhibit the function of other cytokines that use the JAK/STAT pathway of signaling. We have shown, for example, that SOCS1-KIR inhibits type I IFN function, a pathway that utilizes the kinases TYK2 and JAK1 (Waiboci et al., 2007; Ahmed et al., 2009). We have shown here that SOCS1-KIR inhibited IL-23 function, where JAK2 and TYK2 play a role in STAT activation, particularly that of STAT3. We have previously shown that Tkip inhibits IL-6 activation of STAT3, which involves JAK2 (Flowers et al., 2005), and that SOCS1-KIR has a similar effect (Waiboci et al., 2007).

The inhibition of LPS-induced activation of macrophages by SOCS1-KIR demonstrates that it, like SOCS-1, can regulate TLR4 function (Mansell et al., 2006). SOCS-1 regulates TLR4 at multiple sites in macrophage signaling, including MAL, p65 of NF- κ B, an IRAK tyrosine kinase, or induced IFN autocrine activation of the JAKs (Kobayashi et al., 2006). MAL is one of the adapter proteins that are involved in TLR4 signaling (Fitzgerald et al., 2001). Its activation leads, in turn, to the activation of the transcription factor NF- κ B. Enhanced binding of SOCS-1 to activated MAL results in its proteasomal degradation (Mansell et al., 2006). We identified a binding site on MAL for the KIR of SOCS-1, which is consistent with SOCS1-KIR peptide inhibition of TLR4 signaling. We also showed that SOCS1-KIR competes with endogenous SOCS-1 protein for binding to MAL, and that SOCS1-KIR prevents the proteasomal degradation of MAL while inhibiting TLR4 signaling. This would suggest that SOCS1-KIR is able to block MAL function without the need for proteasomal degradation, since SOCS1-KIR lacks the SOCS box. Thus, SOCS1-KIR, like SOCS-1, can inhibit the innate arm of the immune system as well as the IL-23 function that is associated with it.

The therapeutic effectiveness of SOCS1-KIR in the SJL/J model of relapsing/remitting EAE with severe paralysis suggests its potential as a drug for treatment of neurological disorders such as MS, and in other EAE models of MS. We have also shown that Tkip is an effective therapeutic for the C57BL/6 model of chronic EAE (Berard et al., 2010). The use of SOCS

proteins directly as therapeutics in EAE has not been attempted, probably because of the difficulty in obtaining the purified protein in adequate quantities. Another complication is that SOCS proteins act in the cells in which they are produced. There is one report of the use of SOCS-3 protein with an attached cell-penetration sequence in the treatment of sepsis in a mouse model (Jo et al., 2005). Recently, a mutant SOCS-3 protein missing the SOCS box was shown to have a significantly longer half-life in cells treated with inflammatory cytokines compared to wild-type SOCS-3 (Fletcher et al., 2010). This SOCS-3 protein without the SOCS box is functionally similar to our SOCS1-KIR peptide in cell culture. Also, the same group recently reported the use of SOCS-1 protein for the inhibition of STAT1 α activation by IFN γ in cell culture (DiGiandomenico et al, 2009). The specific activity of the SOCS-1 protein was similar to that of SOCS1-KIR.

SOCS-1 and SOCS-3 regulate JAK enzymatic activity by at least two mechanisms. One involves binding to the activation loop of the JAK, and the other involves facilitating proteasomal degradation (Dalpke et al., 2008; Alexander and Hilton, 2004; Yoshimura et al., 2007; Babon et al., 2009; Croker et al. 2008). SOCS1-KIR inhibits JAK function by binding to the activation loop. It is readily produced in quantity through peptide synthesis with an attached palmitate group for cell-penetration. Thus, the rate-limiting conditions that affect the use of SOCS proteins do not apply to the SOCS-1 peptide SOCS1-KIR.

Mice therapeutically treated with SOCS1-KIR showed no infiltration of lymphocytes into the brain. Treatment was initiated 12 days after immunization with MBP, after infiltration of the CNS had begun. The absence of infiltrating lymphocytes at 37 days post-immunization would suggest that SOCS1-KIR reversed ongoing pathology. Th17 cells infiltrating the CNS have recently been shown to produce both IL-17A and IFN γ (Kebir et al., 2009). Cells of similar phenotype were found in relapsing MS patients. We showed here that IL-23 stimulation of MBP-specific lymphocytes resulted in the production of both IL-17A and IFN γ , and that this production was specifically blocked by SOCS1-KIR. SOCS1-KIR also inhibited lymphocyte proliferation as well as the function of both CD4 $^{+}$ and CD8 $^{+}$ T cells. We have previously shown that SOCS1-KIR and Tkip both inhibited IFN γ activation of STAT1 α (Waiboci et al., 2007; Ahmed et al., 2009). It is thought that IFN γ functions to facilitate crossing of the blood-brain barrier (BBB) via the induction of chemokines (Kebir et al., 2009; Cayrol et al. 2008). There are other scenarios of how IFN γ and IL-17A are both involved in the pathology of EAE. Recently it was shown that Th1 cells can compromise the BBB, probably as a result of IFN γ production, and Th17 cells can then enter the CNS and contribute to the pathology of EAE (Kebir et al., 2009). Thus, the inhibition of BBB crossing and CNS infiltration by SOCS1-KIR may be due to the inhibitory effects on IFN γ signaling. In addition to direct inhibition of the effector lymphocytes in EAE, SOCS1-KIR also appears to restore the functional integrity of BBB endothelial cells of EAE mice. These results provide a mechanism for the absence of infiltrating cells in the CNS of EAE mice that have undergone SOCS1-KIR therapy. With respect to the relative, and probably synergistic, roles of Th17 and Th1 cells in MS and EAE, we have shown that SOCS1-KIR inhibits both.

In summary, we have shown that the SOCS-1 mimetic SOCS1-KIR inhibits both the innate and adaptive arms of immune function that are associated with EAE. The inhibition of lymphocyte function at the effector level is consistent with the therapeutic efficacy of SOCS1-KIR, including the reversal of ongoing cellular infiltration of the CNS of EAE mice. The SOCS-1 mimetic appears to compensate for low or modest SOCS-1 and SOCS-3 mRNA expression in the cells associated with the pathology of EAE.

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Figure 1. SOCS1-KIR protects mice from relapsing/remitting EAE

SJL/J mice were injected i.p. with PBS, SOCS1-KIR (60 $\mu\text{g}/\text{mouse}$), or SOCS1-KIR2A (60 $\mu\text{g}/\text{mouse}$) 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. PBS-treated mice had a maximum average disease severity of 4.2, SOCS1-KIR2A-treated mice a maximum average disease severity of 3.4, and SOCS1-KIR-treated mice a maximum average disease severity of 1.8. All mice in the control groups had disease (disease incidence 20 of 20 for PBS-treated mice and 15 of 15 for SOCS1-KIR2A-treated mice) whereas 3 mice in the SOCS1-KIR treated group had no disease (disease incidence 12 of 15) and 8 out of 15 experienced only Stage 1 or less. Statistics were performed using an unpaired Student's t test.

Figure 2. IL-17A production by splenocytes is inhibited in EAE by treatment with SOCS1- KIR
 A) 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 5×10^6 cells/well in RPMI (10% FBS) and incubated 24 hours at 37°C, 5%CO₂. Supernatants were collected and analyzed for IL-17A using the IL-17A Ready-Set-Go ELISA kit (eBioscience, San Diego, CA). B) IL-17A production by splenocytes in response to MBP stimulation is inhibited in mice treated with SOCS1-KIR. Splenocytes were isolated from SJL/J mice 27 days after immunization with MBP. Prior to harvesting the spleens, the mice had been treated as described in part A. Cells were seeded as described above and treated with or without 25 µg/ml MBP and incubated 24 hours. Supernatants were collected and analyzed for IL-17A as in part A. *P < 0.001. C) IL-17A production by splenocytes is inhibited by SOCS1-KIR. Splenocytes were isolated from SJL/J mice 36 days after immunization with MBP. Prior to harvesting the spleens, the mice were scored at EAE stage 1. Cells were seeded as described and peptides were added at the above concentration. After 2 hours, MBP (50 µg/ml) was added to each well and the cells were incubated an additional 24 hours. Supernatants were collected and analyzed for IL-17A as in part A. *P < 0.01, **P < 0.0001. Statistics were performed using two-way ANOVA. The data are representative of three separate experiments

Figure 3. SOCS1-KIR inhibits MBP-induced proliferation of splenocytes

Spleens were harvested from MBP-immunized SJL/J mice, EAE stage 1 (5 weeks after immunization), and 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% CO₂, for 2 hours. MBP (50 μg/ml) 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). *P < 0.05 as determined by two-way ANOVA. The data are representative of three separate experiments.

Figure 4. SOCS1-KIR prevents and reverses lymphocyte infiltration of the CNS during EAE

A) Brain from an untreated mouse 12 days post-immunization with MBP. B) Brain from a naïve mouse. SJL/J mice were injected with (C) PBS, (D) SOCS1-KIR (60 µg/mouse), or (E) SOCS1- KIR2A (60 µg/mouse) every other day beginning 12 days post-immunization with MBP and their brains were collected on day 38 post-immunization. Mice were sacrificed, their brains were collected in 4% PFA in PBS and fixed overnight before being transferred to 70% ethanol. Brains were embedded in paraffin, cut, and stained with H&E. Magnification is 1.25 x; each inset is 20 x.

Figure 5. SOCS1-KIR enters the blood brain barrier endothelial cells of EAE mice but not healthy controls

MBP-sensitized mice at EAE stage 1 (5 weeks after immunization) were injected i.p. with 1 ml (A) FITC-SOCS1-KIR (50 $\mu\text{g}/\text{ml}$) or (B) equivalent FITC alone. After 2 hours, brains were collected in 4% PFA in PBS and fixed overnight before being transferred to 70% ethanol. Brains were then paraffin-embedded, sliced, and sections were dried in the dark for 24 hours. Naïve mice were treated with (C) FITC-SOCS1-KIR or (D) FITC alone and the brains were processed as described. Green = FITC-SOCS1-KIR or FITC. Orange = autofluorescence, mainly due to red blood cells. Magnification is 20 x; inset for A is 40 x.

Figure 6. Expression of SOCS-1 and SOCS-3 mRNA in CD4⁺ T cells of the spleen and CNS of naïve and EAE mice

Spleens and brains were collected from naïve and MBP-sensitized SJL/J mice experiencing EAE at Stage 1 (5 weeks after immunization). Monocytes were isolated from the CNS tissues using a Percoll gradient. CD4⁺ T cells were isolated from single cell suspensions 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 that of the naïve spleen CD4⁺ T cells.

Figure 7. Induction of IL-17A and IFN γ in MBP-sensitized splenocytes by IL-23 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% CO₂, 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, San Diego, CA). *P < 0.05. B) IFN γ 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 IFN γ production using the IFN γ Ready-Set-Go ELISA kit (eBioscience, San Diego, CA). *P < 0.05. Statistics were determined by two-way ANOVA. The data are representative of three separate experiments.

Figure 8. SOCS1-KIR inhibits the production of IFN̳ by CD4⁺ and CD8⁺ T cells

A) CD4⁺ T cells were isolated from the spleens of MBP-immunized mice (4 to 5 weeks after immunization), and incubated with the indicated peptides, inactivated APCs, and MBP before being transferred to IFN̳-ELISPOT plates. Spots indicating IFN̳-producing cells were counted after 48 hours. The number of spots by SOCS1-KIR treated cells, at 11 ̳M, was significantly different from that of the control cells as determined by the Mann-Whitney signed-rank test. **P < 0.001. B) CD8⁺ T cells were isolated and treated as in part A. Spots indicating IFN̳-producing cells were counted after 48 hours. The number of spots by SOCS1-KIR treated cells, at 11 and 33 ̳M, was significantly different from that of the control cells as determined by the Mann-Whitney signed-rank test. *P < 0.01. Samples were run in triplicate and the data are representative of three separate experiments. Viabilities of treated and control cells were similar, approximately 90%.

Figure 9. SOCS1-KIR inhibits enhanced STAT3 activation by IL-23 and IL-17A

A) SOCS1-KIR inhibits IL-23-enhanced STAT3 phosphorylation. Splenocytes isolated from MBP-immunized mice experiencing EAE (stage 1, five weeks after immunization), 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 (100 ng/ml) for 2 hours. Western blots were performed and analyzed as described for part A; however, longer exposure times were necessary. The experiments were performed twice with similar results.

Figure 10. SOCS1-KIR regulates TLR4 signaling

A) SOCS1-KIR inhibits LPS-induced macrophage activity. Murine macrophages (RAW264.7) were incubated with varying concentrations of LPS alone, SOCS1-KIR, or control peptide, at 24 μ M final concentration, for 48 hours. Culture supernatants were collected and nitrite concentration was determined using Griess reagent. There were statistically significant differences between SOCS1-KIR treated cells (0.5 and 1.0 μ g/ml LPS) when compared to the control peptide treated cells as determined by Mann-Whitney signed rank test. * $P < 0.001$. The experiments were performed in triplicate and data are representative of two independent experiments. B) Hydrophobic profiles of murine JAK2 autophosphorylation site with candidate tyrosine phosphorylation site peptides of murine MAL. The autophosphorylation site of JAK2 and MAL(82–94) show similar hydrophobic profiles, while MAL(154–166) candidate site had a different hydrophobic profile. C) SOCS1-KIR binds to a tyrosine phosphorylation site on the TLR4 cytosolic protein MAL. SOCS1-KIR (3 μ g/well) was bound to a 96-well plate and increasing concentrations of biotinylated MAL(82–94), biotinylated MAL(154–166), or biotinylated JAK2 were added. Bound biotinylated peptide was detected with HRP-conjugated neutravidin, followed by the addition of substrate. The reaction was stopped with 2 N H_2SO_4 . Absorbance was read at 490 nm. D) Inhibition of MAL degradation in RAW264.7 macrophages. Cells (2.5×10^6 cells/ml) were treated with LPS (1 μ g/ml) in the absence or presence of SOCS1-KIR, MAL(82–94), or control peptide for the indicated times. Westerns were performed with anti-MAL antibodies. The media lane is a negative control for all treatments, which were completed in one experiment. The data are representative of three independent experiments.

Table 1

Peptide sequences.

Peptide name	Sequence
SOCS1-KIR	⁵³ DTHFRTRSHSDYRRI
SOCS1-KIR2A	⁵³ DTHARTARSHSDYRRI
Tkip	WLVFFVIFYFFR
JAK2	¹⁰⁰¹ LPQDKEYYKVKEP
MAL(82–94)	⁸² WSKD Y DVCVCHSE
MAL(154–166)	¹⁵⁴ DPWCK Y QMLQALT

For binding assays, biotin was added to the following peptides: JAK2, MAL(82–94), and MAL(154–166). For cell penetration, a palmitic acid group was added to the peptides. Bold **Y** indicates the tyrosine is phosphorylated.