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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 IFNγ; 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.

1. Introduction

The immune system is remarkably complex and flexible, composed 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 (IFNγ) 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, 2010; Korn et al., 2009; Boniface et al., 2008; Linker and Lee, 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 and Miller, 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

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short 12-mer peptide, WLVFFVIFYFRR, which binds to the activation loop of JAK2, resulting in inhibition of its autophosphorylation, as well as its phosphorylation of the IFNγ receptor subunit IFNGR-1 (Flowers et al., 2004; Waiboci et al., 2007). This tyrosine kinase inhibitory peptide (Tkip) was developed based on hydropathic 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, ^{53}DTHFRTFRSHSDYRRI (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 IFNγ-induced STAT1α phosphorylation, inhibited IFNγ 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 IFNγ 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 (Szente 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.

Table 1
Peptide sequences.

Peptide name	Sequence
SOCS1-KIR	^{53}DTHFRTFRSHSDYRRI
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 that the tyrosine is phosphorylated.

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

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 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 μl final volume: PBS, SOCS1-KIR (60 μg/mouse), or SOCS1-KIR 2A (60 μg/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 IFNγ production

SJL/J mice were immunized with MBP for EAE induction as described earlier 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 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 h at 37 °C, 5% CO_2. For IL-17A production in response to MBP stimulation, splenocytes were treated with or without 25 μg/ml MBP and incubated for 24 h. 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 earlier. Peptides were added at 0, 3.7, 11, and 33 μM concentrations and cells were incubated at 37 °C, 5% CO_2 for 2 h. MBP was then added to each well at 50 μg/ml and the cells were incubated an additional 24 h. Supernatants were collected and analyzed for IL-17A by ELISA.

In order to determine if SOCS1-KIR can inhibit IL-17A and IFNγ production in response to IL-23, splenocytes from MBP-immunized mice were treated with PBS as described earlier. Peptides were added at 0, 3.7, 11, and 33 μM concentrations and cells were incubated at 37 °C, 5% CO_2 for 2 h before the addition of IL-23 (10 ng/ml). Splenocytes were then incubated an additional 48 h. Supernatants were collected and analyzed for IL-17A as mentioned previously or IFNγ using the IFNγ Ready-Set-Go ELISA kit (eBioscience, San Diego, CA).