Differential expression of suppressors of cytokine signaling-1 and -3 and related cytokines in central nervous system during remitting versus non-remitting forms of experimental autoimmune encephalomyelitis

Jennifer L. Stark and Anne H. Cross

Department of Neurology and Neurosurgery, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8111, Saint Louis, MO 63110, USA

Keywords: cytokines, EAE, multiple sclerosis, SOCS-1, SOCS-3

Abstract

SJL mice exhibit a relapsing–remitting course of experimental autoimmune encephalomyelitis (EAE), whereas C57BL/6 (B6) mice display a more chronic course without complete remissions. Suppressor of cytokine signaling (SOCS)-1 and SOCS-3 are members of a family of inducible intracellular proteins that negatively regulate cytokine signaling in cells of hematopoietic origin and may influence the Th1 to Th2 balance. SOCS-1 and SOCS-3 are induced by cytokines that are known to be up-regulated during EAE, including IFN-gamma (IFN-g) and IL-6, respectively. To test the hypothesis that the level of induction of SOCS-1 and SOCS-3 correlates with the course of EAE, mRNA levels were compared in spinal cords of SJL and B6 mice during discrete stages of disease. SOCS-1 and SOCS-3 were elevated throughout active disease in both strains. At peak EAE, SOCS-1 was higher and SOCS-3 was lower in B6 cords compared with SJL cords. This correlated with greater expression of the Th1 cytokine, IFN-g, and less of the Th2 cytokine, IL-10, in B6 cords relative to SJL cords during onset and peak disease. SOCS-3 inducers in the IL-6 family were expressed differentially between the strains. IL-6 and leukemia inhibitory factor were higher at onset in B6 cords whereas ciliary neurotrophic factor was increased in SJL cords during peak disease. Expression of fibroblast growth factor-2, which may be involved in remyelination, was higher in SJL cords at peak. Comparison of these models suggests that cytokine autoregulatory mechanisms involving SOCS may play a role in determining the course of EAE.

Introduction

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) that can be modeled by immunizing mice with myelin antigens. Most MS patients have a relapsing–remitting form of disease, in which there is nearly complete recovery between attacks, whereas others have progressive forms, in which neurologic impairment accumulates (1). It is not clear what immunological or neurological mechanisms determine disease course. Relapsing–remitting experimental autoimmune encephalomyelitis (EAE) is characterized by functional recovery following acute disease and can be induced by immunizing SJL mice with myelin proteins (2, 3). Immunization of C57BL/6 (B6) mice with myelin oligodendrocyte glycoprotein (MOG)35–55 yields a chronic form of EAE in which complete recovery does not occur (4).

Suppressor of cytokine signaling (SOCS)-1 and SOCS-3 are intracellular proteins that are induced by numerous cytokines and growth factors and regulate inflammatory responses by providing negative feedback to cytokine signaling pathways. SOCS-1 binds to Janus kinases (5), preventing activation of various cytokine signal transduction pathways. Mice lacking SOCS-1 die from organ damage at ~3 weeks of age due to unregulated IFN-gamma (IFN-g) actions (6). SOCS-3 deficiency is a lethal mutation, but studies using macrophages...
lacking SOCS-3 have shown that it negatively regulates the IL-6 pathway by binding to the gp130 receptor (7, 8).

Relative levels of SOCS-1 and SOCS-3 may reflect, as well as influence, the balance of T\(_h1\) and T\(_h2\) cytokines in the local environment. Naive CD4\(^+\) cells express low amounts of both SOCS-1 and SOCS-3, whereas SOCS-1 is elevated in T\(_h1\) cells and SOCS-3 is greatly increased in T\(_h2\) cells (9). In animal models, alteration of SOCS-3 levels can have beneficial or negative effects, depending on the nature of the immune process. Injection of adenoviral vectors containing SOCS-3 into the ankles of mice with antigen-induced arthritis reduced joint swelling, presumably by blocking the pathogenic effects of IL-6 (10). In contrast, SOCS-3 transgenic mice displayed increased severity of a T\(_h2\)-mediated airway hypersensitivity (11). These studies suggest that the relative expression of SOCS-1 and SOCS-3 reflects the balance between T\(_h1\) and T\(_h2\) cytokines, which is thought to be important in regulating the course of EAE and perhaps MS.

The aim of the current experiments was to directly compare the expression of SOCS-1 and SOCS-3 in the spinal cords of SJL and B6 mice during EAE to determine if there was a relationship between SOCS and disease course. B6 CNS expressed relatively more SOCS-1 and less SOCS-3 than SJL CNS during peak disease. This pattern of SOCS expression in the B6 mice correlated with a T\(_h1\) cytokine profile, as well as delayed expression of growth factors that may be involved in remyelination. These molecular findings were associated with differences in EAE course and functional recovery between the two strains.

**Methods**

**Induction and scoring of EAE**

Female SJL/J and C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in microisolater cages. Procedures were approved by the Washington University Animal Studies Committee. EAE was induced in SJL mice by immunizing with 500 \(\mu\)g bovine myelin emulsified (1:1) in CFA containing 60 \(\mu\)g of H37RA Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI). B6 mice were immunized with 10 \(\mu\)g MOG\(_{35-55}\) (Sigma, St Louis, MO) emulsified (1:1) in CFA containing 50 \(\mu\)g H37RA M. tuberculosis. Control animals received injections of CFA without antigen or were age-matched naive. Each mouse received a total of 0.1 ml subcutaneously at two sites (base of neck and tail). Pertussis toxin (List Biological Laboratories, Inc.) was administered intravenously (100–200 ng for SJL and 300 ng for B6) at the time of immunization and 48–72 h later. Mice were scored according to the following scale: grade 0 = no observable neurologic impairments, grade 1 = limp tail, grade 2 = impaired righting reflex when animal is turned onto its back, grade 3 = paralysis of one hindlimb or impairment of both hindlimbs, grade 4 = paralysis of both hindlimbs and grade 5 = moribund or dead. EAE mice that were unable to reach food and water were provided with petri dishes of wet food inside the cage. Mice were sacrificed at different disease stages: pre-clinical = day 7 post-immunization with a grade of 0, onset = within 24 h of first sign of neurologic impairment, peak = grade 3.5–4.0 during acute disease, remission = improvement of at least one grade lasting at least 48 h following acute disease, relapse = worsening of at least one grade lasting at least 48 h following remission and chronic = unchanging clinical score for at least 10 days.

**RNA isolation from spinal cords**

Mice were deeply anesthetized and transcardially perfused with cold diethyl pyrocarbonate (DEPC)-treated PBS. Spinal cords were rapidly dissected, frozen in liquid nitrogen and stored at −80°C until RNA isolation. Each frozen spinal cord was homogenized in 1 ml RNA STAT-60 (TEL-TEST, Friendswood, TX) using a polytron. RNA was isolated according to the RNA STAT protocol, resuspended in DEPC-treated water and stored at −80°C.

**Ribonuclease protection assay**

The RiboQuant Multi-Probe Ribonuclease Protection Assay (RPA) (BD Biosciences, San Diego, CA) was used per manufacturer’s instructions, with a custom mouse template set containing probes for SOCS-1, SOCS-3 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RNA (5 \(\mu\)g) was hybridized with \(\alpha\)-\(\text{\textsuperscript{32}P}\)-labeled probe and protected fragments were run on a urea–polyacrylamide gel. Dried gels were exposed to a phosphor screen (Molecular Dynamics/Amersham, Piscataway, NJ) and analyzed using a STORM 860 phosphorimager (Molecular Dynamics) and the Gel-Pro Analyzer 4.0 program (Media Cybernetics, Silver Spring, MD). The normalized intensity of each band was calculated as a percent of the GAPDH band for the same sample.

**Quantitative (real-time) PCR**

RNA was treated with deoxyribonuclease I (Sigma). cDNA was synthesized using random primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green JumpStart Taq ReadyMix (Sigma) and 1 \(\mu\)l of cDNA template per well. Primer pairs (Sigma-Genosys) for the housekeeping gene GAPDH, as well as the target genes, were synthesized using random primers and SuperScript II reverse transcriptase. Primer efficiency was tested across a range of cDNA concentrations to ensure that it was similar to that of the GAPDH primers. Melting points of the PCR products were determined to confirm a single product. Samples were run in triplicate for 40 cycles to obtain threshold cycle (CT) values for GAPDH and target gene expression. Using 7000 SDS 1.1 Relative Quantification software (Applied Biosystems), target gene expression was first normalized to GAPDH expression. The relative quantitation to a calibrator group, which in this case was SJL naive spinal cords, was then calculated using the equation: \(2^{-\Delta\Delta CT}\). By definition, this results in a value of 1.0 for the calibrator group. The pattern would have been the same if B6 naive cords had been the calibrator.

**Statistical analyses**

Statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA). Clinical EAE courses for SJL and B6 mice were compared by two-way analysis of variance. Student’s \(t\)-tests were used to compare day of disease onset, peak SOCS expression and SOCS-1/SOCS-3 ratio between the two strains. \(P < 0.05\) was considered significant.
Results

Clinical recovery from EAE occurs in SJL mice but not in B6 mice

The clinical courses of EAE for SJL and B6 mice were followed through day 37 post-immunization (Fig. 1). Disease onset occurred earlier in SJL mice than in B6 mice [12.7 ± 1.9 (SD) days post-immunization, n = 19 versus 15.1 ± 3.6 (SD), n = 17; P = 0.01]. Both strains reached similar peak disease scores, but B6 mice had a more severe disease course (P < 0.0001) with continued neurologic dysfunction, while SJL mice recovered from acute disease. Naive, CFA-immunized and pre-clinical mice from each strain, as well as EAE-affected mice at onset, peak, remission and chronic stages of disease, were sacrificed and spinal cords were removed for analysis of gene expression. Table 1 shows the details of the mice studied.

SOCS-1 and SOCS-3 expression in SJL and B6 spinal cords during EAE

To determine if expression of SOCS-1 or SOCS-3 correlated with the different disease courses, spinal cord samples were analyzed by RPA. SOCS-3 mRNA was low and SOCS-1 mRNA was undetectable in naive, CFA-immunized and pre-clinical mice of both strains (Fig. 2A and B). At EAE onset, SOCS-3 was elevated to a similar degree in spinal cords from SJL and B6 mice. In SJL cords, expression of SOCS-3 mirrored active disease, as it remained high at peak, declined during remission and was again highly expressed at relapse. SOCS-3 expression progressively declined from the onset level during peak and chronic EAE in B6 mice. SOCS-1 showed a similar pattern to SOCS-3 in each strain, but was expressed at lower levels. During peak disease, SOCS-1 expression was higher in B6 mice (P = 0.007) and SOCS-3 levels were higher in SJL mice (P = 0.0001).

Th1/Th2 profile in SJL and B6 spinal cords during EAE

SOCS-1 is reported to be preferentially expressed in Th1 cells and SOCS-3 in Th2 cells (9). The SOCS-1/SOCS-3 ratio, which may reflect the relative Th1/Th2 profile, was calculated for SJL and B6 mice during different stages of EAE (Fig. 2C). At disease peak, B6 cords had a significantly greater SOCS-1/SOCS-3 ratio (P < 0.001) than SJL cords, suggesting a more Th1, pro-inflammatory CNS environment. During chronic disease, the ratio in B6 cords dropped to the level seen in cords of sick SJL mice. The ratio was zero in SJL cords during remission due to a lack of SOCS-1 expression.

To assess the cytokine environment, real-time PCR was used to measure expression of IFN-γ, a Th1 cytokine and inducer of SOCS-1, and IL-10, a Th2 cytokine and inducer of SOCS-3. Expression of both cytokines was similar in the control and pre-clinical groups for each strain. During acute disease, remission and relapse, spinal cords from SJL mice (Fig. 3A) showed increased expression of both IFN-γ and IL-10 relative to naive SJL cords. In onset and peak B6 cords, IFN-γ was up-regulated ~4-fold more than SJL cords at the same clinical stage. IFN-γ remained high during chronic disease in B6 mice, whereas IL-10 was only slightly elevated (Fig. 3B).

Expression of IL-6-type cytokines and growth factors during EAE

Since a relative abundance of SOCS-3 mRNA was associated with a better clinical course of disease, real-time PCR was used to quantify the expression of IL-6-related cytokines, which are known to induce SOCS-3 in the CNS (12). These factors were differentially expressed in SJL versus B6 cords during acute disease. At disease onset, IL-6 was greatly

---

Table 1. Summary of mice used in studies

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>SJL</th>
<th>B6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EAE score (number)</td>
<td>Sacrifice day (range)</td>
</tr>
<tr>
<td>Naive</td>
<td>0 ± 0 (4)</td>
<td>13 ± 0.7 (11–14)</td>
</tr>
<tr>
<td>CFA</td>
<td>0 ± 0 (5)</td>
<td>7 ± 0</td>
</tr>
<tr>
<td>Pre-clinical</td>
<td>0 ± 0 (4)</td>
<td>13 ± 1.2 (11–15)</td>
</tr>
<tr>
<td>Onset</td>
<td>3.9 ± 0.1 (5)</td>
<td>14 ± 1.2 (13–19)</td>
</tr>
<tr>
<td>Peak</td>
<td>2.1 ± 0.1 (4)</td>
<td>21 ± 0</td>
</tr>
<tr>
<td>Remission</td>
<td>2.0 ± 0.4 (4)</td>
<td>78 ± 8.6 (61–95)</td>
</tr>
<tr>
<td>Relapse</td>
<td>NA</td>
<td>3.2 ± 0.5 (5)</td>
</tr>
<tr>
<td>Chronic</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Final clinical EAE scores and the day (post-immunization) of sacrifice are shown as mean ± SEM for each group. NA indicates not applicable. The sacrifice day for B6 peak mice occurred before sacrifice of onset mice because the first mice to get sick were not sacrificed at onset but were kept for later disease stages.
up-regulated, with higher expression in B6 cords (Fig. 4A). At peak EAE, IL-6 decreased but remained high in both strains. IL-6 levels declined to near baseline during remission and chronic disease and increased again in SJL cords during relapse. Leukemia inhibitory factor (LIF) displayed a similar expression pattern to IL-6 (Fig. 4B). Ciliary neurotrophic factor (CNTF) expression remained stable in SJL cords until peak disease, when it increased ~2-fold and remained elevated during remission and relapse. Induction of CNTF in cords of B6 EAE mice was delayed until the chronic phase (Fig. 4C). Expression of fibroblast growth factor (FGF)-2, which is induced by CNTF in CNS cells (13) and has been associated with remyelination (14), was increased 3- to 4-fold in SJL cords during peak, remission and relapse stages (Fig 4D). Similar levels of expression in B6 mice were not seen until the chronic phase of EAE.

**Discussion**

The hypothesis underlying these studies was that SOCS-1 and SOCS-3, endogenous negative feedback regulators of cytokine and growth factor actions, play a role in inducing the spontaneous remissions typical of SJL EAE. To address this, CNS expression of SOCS-1 and SOCS-3 in this relapsing–remitting model was compared with that in the chronic, non-remitting B6 model. The largest difference between the two strains occurred during peak EAE. Interestingly, this was immediately prior to the divergence of the clinical courses. At peak, B6 cords displayed higher SOCS-1 and lower SOCS-3 levels compared with SJL cords, resulting in a higher SOCS-1/SOCS-3 ratio. This was associated with greatly elevated expression of the pro-inflammatory cytokine, IFN-γ, which both induces and is regulated by SOCS-1. Compared with SJL CNS, spinal cords from EAE-affected B6 mice at peak disease expressed lower levels of the SOCS-3 inducer, IL-10, as well as of the oligodendrocyte survival factors, CNTF and FGF-2. These data suggest that the failure of B6 mice to completely recover after acute EAE may be due to inadequate production
of SOCS-3 in association with a predominantly pro-inflammatory CNS environment and a delay in expression of neuroprotective factors.

Although there is controversy about the roles of specific cytokines in MS and EAE, our data support the notion that the Th1 cytokine IFN-γ is associated with clinical signs of disease whereas the Th2 cytokine IL-10 may promote recovery from acute EAE. The most striking finding was the extremely high levels of IFN-γ expression in B6 cords from onset through the chronic phase of disease, indicating a Th1-skewed CNS environment. It is likely that the increased expression of SOCS-1 during active disease in both strains was due to this cytokine. SOCS-1 inhibits the functional responses of IFN-γ and other cytokines by inhibiting JAK–STAT pathways (5), but would not necessarily be expected to alter protein or RNA levels of cytokines. In SJL mice, the increase in IFN-γ during active disease was balanced by up-regulated IL-10, resulting in a relatively more Th2-like CNS environment. Increased CNS IL-10 has been implicated in recovery from acute EAE in a number of studies. In an adoptive transfer model of relapsing–remitting EAE, remissions were characterized by increased IL-10 in the spinal cord (15). Furthermore, IL-10-deficient mice do not undergo spontaneous recovery of EAE (16). A recent study found that remission was related to IL-10-secreting CD25+CD4+ regulatory T cells infiltrating the CNS (17).

During EAE, it is likely that localized production of cytokines and growth factors by numerous cell types determines the expression of downstream molecules such as SOCS. Similar to our findings, Maier et al. reported increased expression of SOCS-1 and SOCS-3 in B6 spinal cord and brain. Using in situ hybridization, expression of SOCS was primarily seen in mononuclear cells infiltrating the CNS (18). Other studies have shown that SOCS-1 and SOCS-3 are expressed in the embryonic and adult mouse brain (19) and can be induced in murine astrocytes (20). Therefore, the regulatory pathways activated during EAE may involve both infiltrating and endogenous cells within the complex CNS environment.

The data presented show that a relative abundance of SOCS-3 expression in the spinal cord during peak EAE correlates with remission, but suggest that not all SOCS-3 inducers have the same effects. In various cell types, SOCS-3 is up-regulated by IL-10 (21), as well as IL-6 (22), and the related cytokines, LIF and CNTF (12). In these experiments, higher IL-6 expression in B6 cords at EAE onset did not correlate with improved disease outcome. Members of the IL-6 family share the gp130 receptor, but are known to differentially affect the course of EAE. IL-6-deficient mice are resistant to active induction of EAE (23, 24), perhaps due to a shift towards Th2 cytokine production (25). The presence of endogenous CNTF (26) or exogenous administration of LIF (27) results in less severe EAE and enhanced oligodendrocyte survival. Surprisingly, in the current studies, higher expression of LIF during EAE onset in B6 mice correlated with a poorer disease outcome. This may be due to the fact that over-expression of LIF in the CNS has pro-inflammatory actions (28). In contrast, CNTF has been implicated in the protection of oligodendrocytes from tumor necrosis factor-induced cell death in vitro (29) and in vivo (26). The present studies suggest that induction of SOCS-3 during peak EAE may be involved in subsequent remission. However, more studies must be done.
to clarify whether SOCS-3 is contributing to recovery from acute EAE, or is merely a marker of a favorable cytokine environment.

The combination of elevated CNTF and FGF-2 in spinal cords of SJL mice during peak disease may create an environment which promotes remyelination. CNTF has been shown to enhance myelin formation in vitro (30). Although the role of FGF-2 in remyelination is controversial, it is known to stimulate the proliferation of oligodendrocyte precursors (31). In a murine viral model of demyelination, increased expression of CNTF during the remyelination phase was implicated in the stimulation of spinal cord FGF-2 (13). Increased FGF-2 expression in the same model coincided with recovery of neurologic function (14). Treatment of EAE mice with a vector expressing FGF-2 increased the number of oligodendrocyte precursor cells in damaged areas of the CNS and ameliorated disease (32).

EAE is often used as an animal model for MS, but few studies have taken advantage of the fact that different mouse strains show divergent courses of EAE. We have directly compared a remitting and a non-remitting model of EAE to the distinct disease courses exhibited by MS patients. Further investigations into the differences in EAE course among mouse strains may help us to understand the factors underlying the transition from relapsing--remitting MS to secondary progressive MS, and to develop neuroprotective therapies to complement the currently available immunomodulatory therapies.

Acknowledgements
We are grateful for the excellent technical assistance of Nathan Allen, Bob Mikesell and Michael Ramsbottom. Neville S. Rapp offered helpful suggestions on the manuscript. These studies were funded by the National MS Society (PP0824) and the Barnes-Jewish Hospital. The ABI Prism 7000 was donated for MS research by Bob Mikesell and Michael Ramsbottom. Neville S. Rapp offered helpful suggestions on the manuscript. These studies were funded by the National MS Society (PP0824) and the Barnes-Jewish Hospital. The ABI Prism 7000 was donated for MS research by Bob Mikesell and Michael Ramsbottom.

Abbreviations

- B6: C57BL/6
- CNS: central nervous system
- CNTF: ciliary neurotrophic factor
- C
+ threshold cycle
- DEMP: diethyl pyrocarbonate
- EAE: experimental autoimmune encephalomyelitis
- FGF-2: fibroblast growth factor-2
- GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- IFN-g: IFN-gamma
- IFN-gamma
- LIF: leukemia inhibitory factor
- MOG: myelin oligodendrocyte glycoprotein
- MS: multiple sclerosis
- SOCS: suppressor of cytokine signaling

References

6 Alexander, W. S., Starr, R., Fenner, J. E. et al. 1999. SOCS1 is a critical inhibitor of interferon-gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. Cell 96:597.


