Published in final edited form as:

Exp Neurol. 2010 May ; 223(1): 173–182. doi:10.1016/j.expneurol.2009.06.018.

Differential expression and potential role of SOCS1 and SOCS3 in Wallerian degeneration in injured peripheral nerve

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Abstract

Pro-inflammatory chemokines and cytokines play an important role in Wallerian degeneration (WD) after peripheral nerve injury. These pro-inflammatory signals are “turned-off” in a timely manner to ensure that the inflammatory response in the injured nerve is limited. The factors that regulate the turning-off of the pro-inflammatory state are not fully understood. The suppressors of cytokine signaling (SOCS) proteins are potential candidates that could limit the inflammatory response by acting to regulate cytokine signaling at the intracellular level. In this work we show that the expression SOCS1 and SOCS3 proteins differ from each other during WD in the mouse sciatic nerve after cut/ligation and crush injuries. SOCS1 is mainly expressed by macrophages and its expression is inversely correlated with phosphorylation of JAK2 and STAT3 signaling proteins and the expression of pro-inflammatory cytokines IL-1 β and TNF α . In addition, treatment of cut/ligated nerves, which express lower levels of SOCS1 as compared to crush injury, with a SOCS1 mimetic peptide leads to a decrease in macrophage numbers at 14 days post-injury and reduces IL-1 β mRNA expression 1 day post-injury. In contrast, SOCS3 expression is restricted mainly to Schwann cells and is negatively correlated with the expression of IL-6 and LIF. These data suggest that SOCS1 and SOCS3 may play different roles in WD and provide a better understanding of some of the potential regulatory mechanisms that may control inflammation and regeneration in the injured peripheral nerve.

Keywords

SOCS; Macrophage; Cytokine; Inflammation; Wallerian degeneration; Peripheral nerve

Introduction

Axonal injury induces responses in the distal nerve segment collectively termed Wallerian degeneration (WD) (Stoll et al., 2002; Waller, 1850). An important aspect of this response in peripheral nerves is the recruitment and activation of macrophages that leads to the phagocytosis and clearance of myelin and axonal debris. The rapid clearance of myelin appears to contribute to axon regeneration after peripheral nerve injury because of the presence of axon growth inhibitors in peripheral nerve myelin (Barrette et al., 2008; David et al., 1995; Schafer et al., 1996; Shen et al., 1998). Macrophages are rapidly recruited within 2 days after peripheral

nerve injury, reach maximal numbers at 14 days and are rapidly eliminated thereafter (Mueller et al., 2003; Taskinen and Roytta, 1997).

Several chemokines and cytokines are upregulated after peripheral nerve injury and some of these, such as MCP-1, MIP1 α , IL-1 β , TNF α and GM-CSF have been shown to play a role in mediating macrophage recruitment and activation in WD in peripheral nerves (Be'eri et al., 1998; Boivin et al., 2007; Korompilas et al., 1999; Leskovaar et al., 2000; Liefner et al., 2000; Perrin et al., 2005; Shamash et al., 2002; Subang and Richardson, 2001a; Taskinen et al., 2000; Taskinen and Roytta, 2000; Toews et al., 1998). How this inflammatory response is terminated is not yet fully understood. Members of the IL-6 family of cytokines, such as LIF and IL-6 are also upregulated in the distal portion of the nerve immediately after peripheral nerve injury and contribute to neuronal survival and regenerative responses (Bolin et al., 1995; Bourde et al., 1996; Cafferty et al., 2001; Curtis et al., 1994; Donato et al., 1995; Friedman et al., 1992; Ito et al., 1998; Kirsch et al., 2003; Murphy et al., 1999; Sahenk et al., 1994; Sendtner et al., 1997, 1992; Subang and Richardson, 2001b; Zhang et al., 2004; Zhong et al., 1999).

The suppressors of cytokine signaling (SOCS) proteins are a family of intracellular proteins that have emerged as important intracellular negative regulators of pro-inflammatory cytokine signaling (Alexander and Hilton, 2004; Elliott and Johnston, 2004). Binding of cytokines to their receptors leads to the activation of the JAK/STAT signaling pathway, and transcription of cytokine responsive genes, including the SOCS genes. SOCS proteins inhibit the JAK/STAT pathway, shutting down further cytokine signaling and consequently, their own production (Hilton, 1999; Shuai and Liu, 2003). Different SOCS proteins inhibit JAK/STAT signaling via different mechanisms. SOCS1, for example, binds directly to the activated JAKs on the cytokine receptor via the SH2 domain in SOCS and inhibits its catalytic activity. In contrast, SOCS3 does not bind directly to the JAKs but rather binds directly to the activated cytokine receptor and then inhibits JAK activity (Alexander, 2002). At present, SOCS1, 2, 3 and CIS are the best characterized. Gene targeting studies have demonstrated a critical role for SOCS1 and SOCS3 in the negative regulation of different cytokines. SOCS1 negatively regulates IFN γ , TNF α and IL-1 β (Metcalfe et al., 1999, 2002) while SOCS3 negatively regulates LIF and IL-6 (Crocker et al., 2003; Robb et al., 2005). Recent studies have shown that SOCS3 can limit axon outgrowth in cultured adult rat sensory neurons via negative regulation of gp130 signaling through STAT3 (Miao et al., 2006). At present, the expression and role of SOCS proteins in WD in injured peripheral nerve is not known. In this study, we examined the expression and potential role of SOCS1 and 3 in two types of sciatic nerve injury in mice: (i) cut/ligation injury in which WD occurs but axon regeneration is prevented, and (ii) crush injury in which WD is accompanied by axon regeneration. We show that SOCS1 and SOCS3 are differentially expressed during WD in these two types of peripheral nerve injuries and might play different roles in regulating inflammation and axon regeneration.

Materials and methods

Surgeries

Adult, female BALB/c mice were deeply anaesthetized with ketamine:xylazine:acepromazine (50/10/10 mg/kg) and the left sciatic nerve exposed. For the cut/ligation injury, the nerve was transected and the cut ends ligated to prevent axon regeneration. For the crush injury, the nerve was crushed for 30 s with forceps cooled in liquid nitrogen. Mice from both groups were sacrificed at 1, 3, 7, 14, 21 to 28 days after injury and the nerve segment distal to the lesion was removed. For protein analysis, 8–15 nerves were pooled for each time point because of the small size of the nerves and the small amount of tissue obtained. Nerves were also taken from uninjured animals to serve as controls. All surgical procedures were approved by the

McGill University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

RT-PCR

Total RNA was isolated from sciatic nerves using RNeasy Mini Lipid Tissue kit (Qiagen). Gene-specific primers for TNF α , IL-1 β , LIF, IL-6 and CNTF were designed using PrimerQuest (IDT). RT-PCR was performed using Omniscript RT-PCR kit (Qiagen) according to manufacturer's protocol. The PCR step was performed with the Hotstart Taqman® kit (Qiagen). Peptidylprolyl isomerase (PPIA) or 18S rRNA was used as a control (Feroze-Merzoug et al., 2002). 10–15 nerves were pooled for each time point and the experiments repeated three times.

Western blotting

Nerve samples which included the entire distal segment beyond the lesion site from 3, 7 and 14 days post-injury were pooled and homogenized in 0.5% RIPA buffer (1% NP-40, 1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, 0.02 M EDTA) containing protease inhibitors. After centrifugation, the supernatant was collected, the protein concentration determined, and samples separated by SDS-PAGE on 8–12% gels, and Western blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% milk in PBS-Tween-20 (PBS-T) (0.05%) to prevent non-specific binding of antibodies, and probed with polyclonal rabbit anti-SOCS1 (Zymed, 1:100), anti-SOCS3 (Ab-Cam, 1:100), anti-phospho-JAK2, or anti-phospho-STAT3 (Cell Signaling, 1:100), and reprobed with anti- β -actin (1:200, Sigma). Binding of these primary antibodies was detected with a goat anti-rabbit HRP secondary antibody (Jackson, 1:25,000) and visualized using the Enhanced Chemiluminescence kit (Perkin-Elmer). The pSTAT3 blots gave multiple bands which have also been observed by others using the same antibody (Wang et al., 2009). This may reflect different isoforms of STAT3 specific to the tissue (Biethahn et al., 1999). All experiments were repeated three times. The films were scanned and changes in protein expression quantified with the Image Quant 5.0 software.

Immunofluorescence and confocal microscopy

Mice were deeply anesthetized and perfused with 0.1 M phosphate buffer (pH 7.2) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The nerve distal to the injury was removed, cryoprotected in 30% sucrose and frozen. Longitudinal cryostat sections (10 μ m) were blocked with donkey anti-serum to prevent non-specific binding, and then incubated overnight with a cocktail of two primary antibodies. Rabbit polyclonal antibodies against SOCS1 and 3 (as above) were individually incubated with one of the following cell type-specific monoclonal antibodies against S100 β (Sigma, 1:200; Schwann cell marker) or F4/80 (Serotec, 1:100; macrophage marker). The binding of polyclonal SOCS antibodies was detected with a biotinylated donkey-anti-rabbit secondary antibody (Jackson, 1:400), followed by incubation with Streptavidin-conjugated fluorescein (Jackson, 1:700). Rhodamine-conjugated anti-rat antibody (Jackson, 1:200) was used as a secondary antibody for S100 β and F4/80. For confocal microscopy, a nuclear stain (TOTO; Invitrogen) was used to label nuclei. Immunostained tissue sections were viewed with a spinning disk confocal system (Perkin-Elmer) connected to an Eclipse TE2000 (Nikon).

Quantification

Overlapping single plane confocal images were taken of the nerve distal to the injury at a magnification of 20 $\times$ and used to compile full-length reconstructions. Two tissue sections (at a minimum of 100 μ m apart) were reconstructed per animal and at least five animals were quantified for each condition. Cell counts were done within 3 regions (0.1 to 0.3 mm, 0.5 to 0.7 mm and 0.9 to 1.1 mm) distal to the injury site using the Imaris software (Bitplane). No

differences were found in the percentage of double-labeled cells (see below) at the 3 distances, so these values were pooled. The total number of F4/80 or S100 β positive cells was quantified and the percentage double-labeled with anti-SOCS1 or anti-SOCS3 was estimated. To quantify numbers of SOCS1⁺ cells close to the lesion site, the total cell number was calculated within the region immediately distal to the lesion from 0 to 0.3 mm using DAPI as a nuclear marker and the percentage of cells double-labeled with SOCS1 was estimated.

Administration of SOCS1 mimetic

A SOCS1 mimetic peptide, tyrosine kinase inhibitor peptide (tkip) a short 12-mer peptide, that was previously characterized to bind to the auto-phosphorylation site of JAK2, resulting in inhibition of its auto-phosphorylation and inhibition of phosphorylation of its associated receptor (Flowers et al., 2004), was dissolved in DMSO, diluted in PBS and administered intraperitoneally at a concentration of 63 μ g/mouse, as described previously (Mujtaba et al., 2005). Mice were treated with tkip starting the day of sciatic nerve cut/ligation injury and every other day for a period of 14 days, as this coincided with the peak of inflammation in the nerve. A vehicle control group was administered PBS with an equivalent concentration of DMSO. To assess inflammation following tkip administration, mice were sacrificed immediately following treatment at 14 days. Distal nerve segments were removed and fixed for immunofluorescence analysis as described above. Cryostat sections (10 μ m) of the nerve distal to the cut/ligation were labeled with DAPI and Mac-2, a phagocytic cell marker. The numbers of Mac-2-positive cells distal to the injury site were quantified in two sections 100 μ m apart. Five animals were quantified in the tkip-treated group and 3 animals were quantified for the vehicle-treated group.

Statistical analysis

Statistical analysis was carried out using the Student's t-test. Significance was set at $P < 0.05$.

Results

Expression of SOCS1 and SOCS3 protein after sciatic nerve crush and cut/ligation

We assessed the expression of SOCS1 and SOCS3 by Western blot analysis at 3, 7 and 14 days after crush and cut/ligation injury. After cut/ligation, SOCS1 protein levels were close to the uninjured levels at 3 days, increased about 2 to 3-fold at 7 and 14 days (Figs. 1A, B). In contrast, after crush injury, SOCS1 expression increased rapidly by about 2-fold at 3 days, up to 6-fold at 7 days and remained at 7-fold at day 14 (Figs. 1A, B), which is 3-fold greater than the value in the cut/ligated nerves at 14 days.

In the cut/ligated nerves, SOCS3 expression increased about 19-fold at 3 days and then remained at about 26-fold at 7 and 14 days post-injury (Figs. 1A, C). In striking contrast, after crush injury, SOCS3 expression reached only about 4-fold above uninjured levels at 3 days; continued to increase to 15-fold at 7 days and appeared to decrease slightly to 11-fold at 14 days post-injury (Figs. 1A, C). Interestingly, the expression pattern of SOCS1 and SOCS3 at 14 days was opposite to each other with SOCS1 higher in the crushed nerve and SOCS3 higher in the cut/ligated nerve (Figs. 1A–C).

Cell type-specific localization and quantification of changes in SOCS1 and SOCS3 protein after sciatic nerve crush and cut/ligation

To better understand the differences observed in SOCS1 and SOCS3 expression at 14 days seen by Western blot analysis, we used double-immunofluorescence labeling to assess which cell types express these proteins in the injured nerves. The main cell types in the injured nerve

at 14 days are Schwann cells and macrophages, therefore antibodies against S100 β and F4/80 antigens were used to label Schwann cells and macrophages, respectively.

SOCS1

In both cut/ligated and crushed nerves, SOCS1 was expressed predominantly by F4/80⁺ macrophages (Figs. 2A–C). In the cut/ligated nerve, 15% $\pm$ 1.6 of F4/80⁺ cells expressed SOCS1, while in the crushed nerve significantly fewer (8.6% $\pm$ 1.3) F4/80 cells were SOCS1⁺ (Figs. 2A, B, E). In the cut/ligated nerve, SOCS1 was expressed exclusively by macrophages and this pattern was maintained along the entire distal length of the nerve (Fig. 2A). However, in crush nerves, SOCS1 immunoreactivity was also observed in S100 β ⁺ Schwann cells in the region directly adjacent to the injury site (Fig. 3). Quantification of the SOCS1⁺/S100 β ⁺ cells at the site of injury (i.e., 300 μ m distal to the injury) at 14 days showed that 26.4% $\pm$ 0.02 of the Schwann cells expressed SOCS1 in the crush-injured nerve, while in the cut/ligated nerve none of the Schwann cells expressed SOCS1.

SOCS3

In contrast to SOCS1, SOCS3 was expressed predominantly in Schwann cells along the distal portion of the injured nerves. In the cut/ligated nerve, 43% $\pm$ 5.4 of the Schwann cells were SOCS3⁺, while in the crushed nerve, only 14.8% $\pm$ 2.1 of the S100 β ⁺ Schwann cells were SOCS3⁺ (Figs 4A–E).

JAK2 and STAT3 activation after sciatic nerve crush and cut/ligation

As SOCS1 and SOCS3 have been reported to inhibit cytokine signaling through the JAK2/STAT3 pathway (Crocker et al., 2003; Metcalf et al., 1999; Sakamoto et al., 2000; Starr et al., 1997) we next assessed JAK2, STAT3 phosphorylation in the sciatic nerve after crush and cut/ligation injury. To correlate phosphorylated JAK2 and STAT3 levels with SOCS protein expression, we did Western blots with tissues at the same time points of 3, 7 and 14 days. Phosphorylated JAK2 (pJAK2) was detected at low levels in the uninjured nerve and this was not significantly changed after cut/ligation at any time points (Figs. 5A,B). After crush injury, pJAK2 was increased about 3-fold at 3 days after injury (Figs. 5A, B). On the other hand, phosphorylated STAT3 (pSTAT3) was also detected at very low levels in the uninjured nerve, started to increase in the cut/ligated nerves at 3 and 7 days and increased further to about 8.5-fold at 14 days (Figs. 5A, C). In contrast, in the crushed nerves, pSTAT3 levels were much higher (13-fold) at 3 days and decreased thereafter at 7 days (6-fold) and 14 days (3-fold) post-injury (Figs. 5A, C). As expected, the levels of pJAK2 and pSTAT3 appeared to be negatively correlated with SOCS1 levels (Figs. 1B and 5B). There was also a negative correlation with SOCS3 and pSTAT3 levels after crush injury (Figs. 1C and 5C).

The primary Janus associated kinase that SOCS1 binds to is JAK2 (Yasukawa et al., 1999). Double immunofluorescence labeling of the distal nerve at 14 days after cut/ligation injury showed that pJAK2 and pSTAT3 were localized in macrophages (Figs. 5D–I), which were also shown above to express SOCS1 (Fig. 2). These results suggest that SOCS1 may inhibit the JAK2/STAT3 pathway in macrophages. It is possible that SOCS3 may regulate other JAK/STATs, such as JAK1/STAT1 but we could not detect these using the available pJAK1 or pSTAT1 antibodies (Cell Signaling).

Cytokine mRNA expression after sciatic nerve crush and cut/ligation

The expression of SOCS1 in macrophages and SOCS3 in Schwann cells suggest that these SOCS proteins are likely to play distinctly different roles in WD. One possibility is that SOCS1, which is expressed in macrophages, regulates inflammation, while SOCS3, which is expressed in Schwann cells, contributes to the regulation of cytokines involved in axon regeneration. To

address this possibility, we assessed by RT-PCR the mRNA expression profile of key cytokines involved in regulating inflammation and regeneration.

Expression of IL-1 β and TNF α mRNA

Pro-inflammatory cytokines IL-1 β and TNF α are known to be expressed early after sciatic nerve cut and crush injuries (Leskovaar et al., 2000; Perrin et al., 2005; Shamash et al., 2002; Taskinen et al., 2000) and play a role in recruiting macrophages into the nerve (Liefner et al., 2000; Perrin et al., 2005). However, their expression patterns over the course of WD in a cut/ligation versus a crush injury have not been directly compared. We observed little if any IL-1 β expression in uninjured nerves. In the cut/ligated nerve, IL-1 β expression increased by 30-fold at 1 day post-injury, then decreased at 3 days, followed by a second increase in expression starting at 7 days (19-fold) and increasing further at 14 days (24-fold) and 21 and 28 days (about 44-fold) (Figs. 6A, B). In contrast, in the crushed nerve, IL-1 β mRNA was detected at very low levels after injury, with a peak at 28 days (Figs. 6A, B).

TNF α mRNA was also observed at very low levels in uninjured nerves (Figs. 6A, C). In the cut/ligated nerve, TNF α mRNA expression showed a similar pattern to IL-1 β (Figs. 6A, C). In the crushed nerves, TNF α mRNA levels increased rapidly at 1 day after injury (3-fold), were maintained until day 14, and decreased thereafter (Figs. 6A, C). The greater and prolonged expression of IL-1 β and TNF α in the cut/ligated nerve from 14 days post-injury (Figs. 6B, C) correlates with the more extensive inflammation observed in this injury model and the low expression of SOCS1 at 14 days post-injury.

Expression of IL-6, LIF and CNTF mRNA

In uninjured nerve, neither LIF nor IL-6 mRNA were expressed (Figs. 6A, D, E). In cut/ligated nerves, IL-6 mRNA was increased (13-fold) at 1 day post-injury and decreased rapidly thereafter, while LIF mRNA displayed a modest peak at 1 day (4.5-fold) and was barely detectable at later time points after injury (Figs. 6A, D, E). In contrast, in crushed nerves, there was a marked increase IL-6 mRNA at 1 day (30-fold) and 3 days (12-fold) post-injury (Figs. 6A, D) and in LIF mRNA at day 1 (19-fold) (Figs. 6A, E). In the cut/ligated nerve in which regeneration does not occur, the expression of CNTF was minimal at all time points, while in the crushed nerves which regenerate, its expression remained at normal levels at day 1, decreased thereafter but increased again at day 21 and was restored to normal levels by 28 days (Figs. 6A, F). At 3, 7 and 14 days in both types of injuries, the expression of SOCS3 protein was negatively correlated with the expression of these neuropoietic cytokines.

Effect of SOCS1 mimetic peptide (tkip) after sciatic nerve cut/ligation

As mentioned above, SOCS1 expression is lower in the cut/ligated nerves at 14 days compared to the crush-injured nerves (Figs. 1A, B). This is correlated with greater inflammation in the cut/ligated nerve, as reflected by the greater number of macrophages (Fry et al., 2007) as well as the increased and prolonged expression of pro-inflammatory cytokines IL-1 β and TNF α (Fig. 6). We therefore hypothesized that the decreased expression of SOCS1 between 10 and 14 days might underlie the greater number of macrophages in the cut/ligated nerves. To address this possibility, we treated mice for the first 14 days after cut/ligation injuries with tkip, a SOCS1 mimetic peptide. We found a ~30% reduction in the number of macrophages in the distal nerve segment of mice treated with tkip as compared to those treated with vehicle (Figs. 7A–C; $P=0.004$). Further analysis showed that treatment with tkip significantly reduced IL-1 β mRNA expression by 57% as compared to vehicle-treated cut/ligated nerves, one day after cut/ligation (Figs. 7D and E).

Discussion

In this study, we characterized the expression of SOCS1 and SOCS3 in the adult mouse peripheral nerve undergoing WD after either cut/ligation or crush injuries. We show that (i) SOCS1 is mainly expressed by macrophages, while SOCS3 is expressed by Schwann cells; (ii) SOCS1 protein expression at 14 days is about 3-fold higher in the crushed nerves than in the cut/ligated nerves, while SOCS3 expression shows the opposite pattern; (iii) SOCS1 expression is inversely correlated with phosphorylation of JAK2 and STAT3, and the expression of pro-inflammatory cytokines IL-1 β and TNF α ; (iv) SOCS3 expression is negatively correlated with the expression of IL-6, LIF and CNTF; (v) Treatment with a SOCS1 mimetic peptide reduces IL-1 β mRNA expression and the number of macrophages in the distal degenerating nerve segment after cut/ligation injury. These results show that the expression of SOCS1 and SOCS3 are differentially regulated following sciatic nerve cut/ligation and crush injuries and suggest that they may play different roles in Wallerian degeneration and subsequent axon regeneration.

SOCS1 may regulate macrophage responses in the injured peripheral nerve

There is growing evidence that SOCS1 plays a critical role in regulating inflammatory and immune responses. SOCS1 knockout (SOCS1^{-/-}) mice die at 3 weeks of age as a result of severe lymphopenia, fatty degeneration of the liver, activation of peripheral T cells and infiltration of major organs by T cells and macrophages (Fujimoto and Naka, 2003; Starr and Hilton, 1998). SOCS1 appears to have a protective role in inflammatory diseases such as acute inflammatory arthritis (Egan et al., 2003) and inflammatory bowel disease/colitis (Chinen et al., 2006). SOCS1 is also involved in the regulation of the innate immune response, e.g., LPS and CpG-DNA can induce SOCS1 expression in macrophages, and SOCS1 negatively regulates LPS-induced macrophage activation (Alexander and Hilton, 2004; Johnston, 2004; Kinjyo et al., 2002; Kubo et al., 2003). However, at present, little is known about the role of SOCS in regulating inflammatory responses in the nervous system. SOCS1 was reported to have protective effects in experimental autoimmune encephalomyelitis (EAE), a widely used animal model of multiple sclerosis, in which the SOCS1 mimetic (tkip) was shown to prevent relapses (Mujtaba et al., 2005).

The inflammatory response after peripheral nerve crush injury is tightly controlled. Macrophage numbers and phagocytic activity peak in the first two weeks after injury, and then decline toward normal levels (Mueller et al., 2003; Taskinen and Roytta, 1997). In contrast, cut/ligation injury results in more severe and prolonged inflammation. We have recently shown that there are greater numbers of macrophages in the cut/ligated nerves than in crushed nerve at 14 days (Fry et al., 2007). We now show that there is prolonged expression of the pro-inflammatory cytokines IL-1 β and TNF α in the cut/ligated but not crushed nerves, which also correlates with decreased expression of SOCS1. SOCS1 is known to regulate IFN γ signaling, however, we did not detect any increase in IFN γ expression in injured peripheral nerves in our previous work (Perrin et al., 2005). It is possible that SOCS1 may regulate other cytokines. In support of this, SOCS1^{-/-}/IFN γ ^{-/-} double knockout mice are only partially rescued and succumb to a range of inflammatory diseases later in life (Metcalf et al., 2002). In fact, there are many additional inflammatory cytokines, including those that do not classically signal through the JAK/STAT pathway, that have been shown to be regulated by SOCS1 including TNF α , IL-1, IL-4, IL-8 and IL-10 (Chong et al., 2005; Dickensheets and Donnelly, 1999; Ding et al., 2003; Hanada et al., 2003; Starr et al., 1997; Yoshimura et al., 2004). There is evidence that SOCS1 inhibits constitutive and IL-6 induced activation of STAT3 in non-CNS cells (Flowers et al., 2005). We found a correlation between SOCS1 protein levels and diminished pJAK2/pSTAT3 levels in the injured nerves. In addition, we found that SOCS1, pJAK2 and pSTAT3 were specifically localized in macrophages. Therefore, SOCS1 may inhibit the action

of cytokines that signal through JAK2/STAT3 pathway, such as G-CSF and IL-6 (Johnston, 2004) that are known to activate macrophages, in addition to inhibiting via non-classical JAK/STAT signaling pathways in macrophages, as noted above. At present, very little is known about how SOCS1 inhibits such pathways, but it may involve crosstalk between different cytokine and chemokine signaling pathways (Hilton, 1999; Yoshimura et al., 2003). Recent studies indicate that SOCS1 can negatively regulate Toll-like receptor (TLR) signaling, particularly via TLR-2 and 4 (Mansell et al., 2006). This is interesting in the context of peripheral nerve injury, as there is evidence that IL-1 β expression and the recruitment and activation of macrophages is reduced in the degenerating distal segment of injured nerves in mice lacking TLR2 and TLR4 (Boivin et al., 2007). The macrophages in the injured nerve have been shown to be cells largely recruited from the peripheral circulation (Bendszus and Stoll, 2003).

In this study, we found that SOCS1 protein expression by Western blot analysis is higher in the crushed versus cut/ligated nerves at 14 days. Although the number of macrophages that express SOCS1 distal to the site of injury is lower in the crushed nerves, there is, however, a high level of expression of SOCS1 in Schwann cells near the site of injury, which may account for the higher levels seen by Western blot analysis. This up regulation of SOCS1 at 14 days in the crushed nerve may suppress cytokine/chemokine signaling and thus reduce the number of macrophages in the later stages of WD. On the other hand, the lower level of SOCS1 expression in the cut nerve may underlie the on-going expression of chemokines/cytokines that may contribute to the increased numbers of macrophages after this type of nerve injury.

We also provide evidence that administration of the SOCS1 mimetic, tkip, during the first 14 days after cut/ligation significantly reduces the number of Mac-2⁺ phagocytic cells in the distal nerve segment. This data suggests that SOCS1 activity could play a role in controlling or reducing the inflammatory response during WD in the injured sciatic nerve. It also suggests that the endogenous level of SOCS1 is not sufficient to substantially reduce the number of macrophages after cut/ligation. Our previous work also suggested that in addition to macrophages not leaving the nerve, there might be continued influx of macrophages in the cut/ligated nerve (Fry et al., 2007). It is possible that the on-going high expression of proinflammatory cytokines in the cut/ligated nerve may contribute to the influx of these cells (Perrin et al., 2005).

SOCS3 may regulate regenerative responses after peripheral nerve injury

SOCS3 is implicated in the negative regulation of the IL-6 family of neuropoietic cytokines that include LIF, CNTF and IL-6. SOCS3 knockout mice are embryonic lethal due to placental defects, owing largely to unbridled LIF signaling (Robb et al., 2005). Conditional SOCS3 gene targeting studies have demonstrated additional roles for SOCS3 in the negative regulation of IL-6/gp130 signaling in macrophages (Croker et al., 2003; Lang et al., 2003). There are no reports about the expression and potential role of SOCS3 in Wallerian degeneration in peripheral nerves.

Recent work has demonstrated that SOCS3 expressed in primary sensory neurons can suppress STAT3 mediated neurite growth *in vitro* (Miao et al., 2006). In the present study, we found that SOCS3 is upregulated in the distal segment of peripheral nerves following both cut/ligation and crush injuries, and is selectively expressed by Schwann cells. Our data suggest that SOCS3 is likely to play a different role in the injured nerve as compared to SOCS1, which is expressed by macrophages. The changes in the expression of the IL-6 family of cytokines (IL-6, LIF and CNTF) that we observed were similar to those reported previously (Ito et al., 1998). CNTF which is expressed in the normal nerve is rapidly down-regulated after injury and re-expressed at normal levels after regeneration occurs, while LIF and IL-6 which are not expressed at detectable levels in the normal nerve are increased transiently early after injury. The increased

expression of SOCS3 in Schwann cells in the lesioned nerves that we have seen could likely be induced by LIF and IL-6 and SOCS3 has been shown to negatively regulate signaling via these two cytokines (Croker et al., 2003; Robb et al., 2005). Although these cytokines play a role in neuronal survival and regenerative responses after injury, their prolonged high levels of expression may lead to detrimental inflammatory responses (Lacroix et al., 2002). For example, increased stimulation of gp130, the signal transducing receptor subunit of the IL-6 family with hyper-IL-6 (consisting of IL-6 and its α receptor) in the rat spinal cord led to a marked influx of neutrophils and macrophages (Lacroix et al., 2002). The increased inflammation seen under this condition was detrimental to tissue viability and repair (Lacroix et al., 2002). The negative correlation of IL-6 and LIF expression with SOCS3 protein expression at 3, 7 and 14 days following both cut/ligation and crush injuries suggests that the increased expression of SOCS3 may reduce expression of these cytokines. There is in fact *in vitro* evidence that stable transfection of SOCS3 in a macrophage cell line down-regulates IL-6 mRNA expression (Berlato et al., 2002). In addition, SOCS3 is also known to reduce signaling via IL-6 (Croker et al., 2008). SOCS3 could thus help via such mechanisms to keep the inflammatory process during Wallerian degeneration in check. The higher levels of SOCS3 in the cut/ligated nerve at 7 and 14 days post-injury as compared to the crushed nerve may be an attempt by the cut nerve to curb the inflammatory response in an injury model in which the number of macrophages is greater (Fry et al., 2007). There are no SOCS3 mimetics currently available, and SOCS3 knockouts are not viable. Therefore, it is not possible at present to obtain direct evidence of the role of SOCS3 in peripheral nerve injury. Conditional knockouts in Schwann cells would also be a useful tool to study the role of SOCS3 in Schwann cells in the injured nerve.

The data presented here suggest that SOCS1 and SOCS3 may be involved in different aspects of WD after peripheral nerve injury, and provide new insights into some of the possible mechanisms that may control inflammation and regenerative responses in the injured peripheral nerve.

Acknowledgments

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR; MOP-14828) to SD. EIG and DB are supported by an MS Society of Canada studentship and postdoctoral fellowship, respectively. MH is supported by a CIHR studentship. HMJ is supported by Grant R01 NS 051245 and R01 AI 056152 from the National Institutes of Health (NIH). We thank R. Lopez-Vales for helpful suggestions on the manuscript and M. Attiwell for help with the illustrations.

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Fig. 1. Differential expression of SOCS1 and SOCS3 protein in cut/ligated and crushed nerves. (A) Western blots showing changes in levels of SOCS1 and SOCS3 protein in the distal portion of the sciatic nerve at different times after cut/ligation and crush injury. β -Actin was used as a loading control. (B, C) Graphs showing the quantification of the Western blot data for SOCS1 (B) and SOCS3 (C) (mean $\pm$ SD, $n = 3$; * $P < 0.05$ significantly different from uninjured nerve (ui); # $P < 0.05$ significantly different between cut versus crush). Note that SOCS1 expression is higher in the crush than in the cut/ligated nerve, while the opposite occurs with SOCS3.

Fig. 2.

SOCS1 is expressed by macrophages in the cut/ligated and crush-injured sciatic nerve at 14 days post-injury. Montage of longitudinal sections of cut/ligated (A) and crushed (B) nerves at 14 days post-injury showing double-immunofluorescence labeling with anti-SOCS1 (green), monoclonal antibody F4/80 (red). The nuclei are stained with TOTO (blue). Proximal is to the left and distal is to the right; the lesion site lies at the left of the image. Note that in the cut/ligated nerve (A) expression of SOCS1 is exclusively localized to F4/80⁺ macrophages. In contrast, in the crushed nerve (B) SOCS1 is expressed by F4/80⁺ macrophages that are abundant distally along the nerve, as well as in elongated profiles closer to the site of lesion that are shown to be Schwann cells (see Fig. 3). (C and D) Higher magnification single plane confocal images of the cut/ligated nerves show that SOCS1 is expressed in F4/80⁺ macrophages (arrowheads) (C) but not in S100 β ⁺ Schwann cells (D) in the more distal portion of the nerve. Arrowheads in panel D point to SOCS1⁺/S100 β ⁻ cells. Nuclei are labeled with TOTO (blue). (E) Quantification of the percentage of F4/80⁺ macrophages that are SOCS1⁺ shows a significantly greater number of SOCS1⁺/F4/80⁺ cells in the cut/ligated nerves as compared to

the crushed nerves (mean $\pm$ SD, n = 5; * P <0.05). Scale bars: A, B = 100 μ m; C, D = 10 μ m.
(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. SOCS1 is expressed by Schwann cells close to the lesion site in crush-injured nerves. Single plane confocal images of the region immediately distal to the crush site 14 days after injury show that SOCS1 (A) is expressed in S100 β ⁺ Schwann cells (B). (C) Shows the merged image; nuclei labeled with TOTO. The areas outlined in the white box are shown at higher magnification in panels D–F. Arrowheads in D–F indicate SOCS1⁺/S100 β ⁺ Schwann cells. Scale bars: C = 100 μ m; F = 20 μ m.

Fig. 4. SOCS3 is expressed exclusively by Schwann cells. Montage of the distal segment of the cut/ligated (A) and crushed (B) sciatic nerves 14 days post-injury, showing double-immunofluorescence labeling with anti-SOCS3 (green) and S100β (red) antibodies. Nuclei are stained with TOTO (blue). Note that SOCS3⁺/S100β⁺ cells are more abundant in the cut/ligated nerves (A). (C) Single plane confocal image of SOCS3⁺/S100β⁺ Schwann cells (arrowheads). Panel D shows that SOCS3 is not expressed by F4/80⁺ macrophages. Arrowheads indicate SOCS3⁺ cells. (E) Quantification of the percentage of SOCS3⁺/S100β⁺ Schwann shows that there is a significantly greater number of these cells in the cut/ligated nerves as compared to the crushed nerves (mean±SD, n = 5; **P*<0.05). Scale bars: A, B = 100 μm; C, D = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Differential regulation of pJAK2 and pSTAT3 after cut/ligation and crush injuries. (A) Western blot of phosphorylated forms of JAK2 and STAT3 with β -actin as loading controls. pJAK2 is only present at 3 days following a crush injury. pSTAT3 levels increase over time in the cut/ligation injury, but are highest at 3 days then decline at 7 and 14 days in the crush injury. (B, C) Graphs showing the quantification of the Western blot data for pJAK2 (B) and pSTAT3 (C) (mean $\pm$ SD, n=3; * P <0.05 significantly different from uninjured nerve (ui); # P <0.05 significantly different between cut versus crush). (D–F) Double immunofluorescence labeling showing that pJAK2 is expressed by F4/80⁺ macrophages in the distal portion of the crush-injured nerve. (D) pJAK2 stain; (E) F4/80 stain; (F) merged image showing pJAK2;F4/80;DAPI labeling. (G–I) Double immunofluorescence labeling showing pSTAT3 is also expressed by macrophages in the distal portion of the crush-injured nerve. (G) pSTAT3 stain; (H) F4/80 stain; (I) merged image showing pJAK2;F4/80;DAPI labeling. Scale bars in I = 30 μ m.

Fig. 6.

Changes in mRNA expression of pro-inflammatory and neuropoietic cytokines following cut/ligation and crush injury. (A) RT-PCR showing changes in mRNA expression of IL-1 β , TNF α , IL-6, LIF and CNTF in uninjured (ui), cut/ligated and crushed nerves at 1, 3, 7, 14, 21 and 28 days normalized to PPIA. (B–F) Quantification of the RT-PCR data for IL-1 β (B), TNF α (C), IL-6 (D), LIF (E) and CNTF (F) (mean $\pm$ SD, $n = 3$; * $P < 0.05$ significantly different from uninjured nerve (ui); # $P < 0.05$ significantly different between cut versus crush). The expression of IL-1 β and TNF α is more pronounced in the cut/ligated nerve than the crushed nerve. IL-6 and LIF expression is upregulated immediately after crush, but not cut injury. CNTF mRNA is expressed in the naive nerve and is rapidly down-regulated after cut/ligation and crush injuries. Its levels are fully restored in the crushed nerves by 28 days but remain low in the cut/ligated nerves.

Fig. 7.

Tkip reduces the number of Mac-2⁺ cells in the injured distal nerve at 14 days. Merged image showing immunofluorescence staining for Mac-2⁺ macrophages in the cut/ligated nerve 14 days after injury. (A) Vehicle-treated group, (B) Tkip-treated group. Note that there are fewer Mac-2⁺ cells in the tkip-treated nerve (B) as compared to the vehicle-treated one (A). (C) Quantification shows a significant reduction in the number of Mac-2⁺ cells in the tkip-treated versus the vehicle-treated nerves (mean $\pm$ SD, n = 5 (tkip group) and n = 3 (vehicle group); * P <0.05). Scale bar in B = 30 μ m. (D) RT-PCR data showing differences in mRNA expression of IL-1 β in uninjured (ui), vehicle-treated (veh), and tkip-treated (tkip) cut/ligated nerves at one day after injury, normalized to 18S rRNA. Note the reduction in expression in the tkip-treated group as compared to vehicle-treated control. (E) Quantification of the RT-PCR data illustrated in panel D (n = 3). As expected, the lesion induces a marked increase in IL-1 β mRNA expression as compared to the uninjured nerve (ui versus veh). Note the significant reduction in expression in the tkip-treated group as compared to the vehicle-treated group; * P <0.05.