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Molecular Mechanism of Lipopolysaccharide-Induced SOCS-3 Gene Expression in Macrophages and Microglia

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Immunological activation of macrophages/microglia within the CNS leads to the production of cytokines and chemokines that ultimately impact on glial and neuronal function. Suppressor of cytokine signaling (SOCS) proteins are negative regulators of adaptive and innate immune responses. Our previous studies demonstrated that SOCS-3 attenuates macrophage/microglial activation in vitro, suggesting that SOCS-3 may exert beneficial effects for immune-mediated CNS diseases in vivo. In this study, we describe LPS as a potent inducer of SOCS-3 transcription and expression in macrophages/microglia. An analysis of the SOCS-3 promoter indicates that AP-1 and IFN-γ activation sequence (GAS) elements are involved in LPS-induced SOCS-3 transcription. LPS-induced SOCS-3 expression was diminished in IL-10-deficient macrophages at later time points, indicating the involvement of endogenous IL-10 in this response. Blocking STAT-3 expression and activation using STAT-3 small interfering RNA reduced LPS-induced SOCS-3 gene expression. LPS activated the MAPK-ERK1/2, JNK, and p38 pathways that, in addition to STAT-3, were also involved in LPS-induced SOCS-3 expression. LPS treatment of cells led to the acetylation of histones H3 and H4 on the SOCS-3 promoter and the recruitment of STAT-3, c-Jun, c-Fos, CREB-binding protein, p380, and RNA polymerase II to the endogenous SOCS-3 promoter in a time-dependent manner. These results indicate that LPS-induced MAPK activation, the production of endogenous IL-10, and STAT-3 activation play critical roles in SOCS-3 expression, which provides feedback attenuation of cytokine-induced immune and inflammatory responses in macrophages and microglia. The Journal of Immunology, 2007, 179: 5966–5976.

Cytokine signaling pathways are negatively regulated by the family of proteins called suppressor of cytokine signaling (SOCS) (1–3). These constitute a family of eight related members, SOCS-1 to -7 and cytokine-inducible Src homology 2 (SH2)-containing protein (CIS), which are characterized by the presence of a central SH2 domain, an N-terminal domain of variable length, and a C-terminal conserved domain termed the SOCS box (1). In addition, SOCS-1 and SOCS-3 have a kinase inhibitory region (KIR) in their N-terminal domain. SOCS proteins are inducible by a number of stimuli and function to inhibit signaling through the JAK-STAT pathway in a classic negative feedback loop, using a variety of mechanisms for inhibition (4). The SOCS-3 protein is critical in regulating signaling by the IL-6 family of cytokines by inhibiting STAT-3 activation (5, 6). Recent studies suggest that SOCS-3 has a broader spectrum of action in that it can positively regulate the ERK-MAPK pathway (7), inhibit the NF-κB pathway (8), and antagonize cAMP-mediated signaling (9). SOCS-3 deficiency is embryonic lethal due to defects in placental development by an enhanced activation of the LIF signaling pathway (10). Targeted deletion of SOCS-3 in macrophages results in markedly enhanced IL-6-induced STAT-3 activation (11). Mice deficient in SOCS-3 in hematopoietic and endothelial cells demonstrate exacerbated IL-1-dependent arthritis (12). Conversely, forced expression of SOCS-3 in mouse arthritis models suppressed the induction and/or development of disease (1, 13). Furthermore, intracellular administration of a cell-penetrating SOCS-3 suppressed the cytokine-mediated signal transduction associated with acute inflammation (14). These data collectively indicate that SOCS-3 is a negative regulator of inflammatory responses.

A large number of stimuli are able to induce SOCS-3 expression in a cell type-specific manner, including erythropoietin, IFN-γ, IFN-β, LPS, oncostatin M, growth hormone, IL-4, IL-21, IGF, IL-27, LIF, IL-6, and cAMP (15–23). LIF activation of STAT-3 leads to SOCS-3 expression in corticotroph AtT-20 cells (16). IL-4 induction of SOCS-3 gene expression in B cells occurs by activation of the p38 MAPK pathway (15), whereas IL-8 induction of SOCS-3 occurs in a STAT-independent manner in human myeloid cells (24). Interestingly, the peroxisome proliferator-activated receptor γ (PPAR-γ) agonists 15d-PGJ2 and rosiglitazone induce SOCS-3 expression in primary astrocytes and BV2 microglial cells in a STAT-1-independent manner with the possible involvement of protein kinase A/protein kinase C (25). These studies demonstrate that the activation of STAT-3, p38 MAPK, and/or STAT-independent pathways leads to SOCS-3 gene expression in a stimulus- and cell type-specific manner. Furthermore, a recent study demonstrated that TNF-α...
regulates SOCS-3 expression by stabilization of SOCS-3 mRNA, which occurs by activation of the p38 MAPK pathway (26).

LPS, a Gram-negative cell wall component recognized by the receptor TLR4, triggers innate immune responses in macrophages and microglia (27–29). The TLR family signals via shared downstream signaling molecules including the adapter molecule MyD88, activating NF-κB and MAPKs. Both the NF-κB and MAPK pathways are critical for the induction of numerous immune response genes including cytokines and chemokines (27, 30). In addition, there are also MyD88-independent pathways that induce a number of IFN-β-dependent genes through the induction of endogenous IFN-β to activate the JAK-STAT pathway (31–33). We have recently shown that LPS induces CD40 gene expression in macrophages and microglia, which involves the activation of NF-κB, the induction and activation of IFN regulatory factor 3 (IRF-3), and the subsequent endogenous production of IFN-β that induces STAT-1α activation (34). Furthermore, the SOCS-3 protein attenuates LPS-induced CD40 expression by inhibiting LPS-induced STAT activation (35).

SOCS-3 has a nonredundant role in inhibiting IL-6- and gp130-mediated signaling in a variety of cell types, as well as other signaling pathways (5, 6, 11). Proinflammatory stimuli such as bacterial products (LPS and CpG) (35–38) are potent inducers of SOCS-3 in a variety of cell types. Thus, with respect to regulating the functional effects of IL-6 cytokine family members, it is important to elucidate the molecular mechanisms involved in SOCS-3 gene induction. The results from this present study indicate that LPS stimulation evokes a complex series of signal transduction pathways (STAT-3, ERK1/2, p38 MAPK, and JNK MAPK), histone modifications, and the stepwise recruitment of transcription factors and regulators to the SOCS-3 promoter to coordinate the expression of the SOCS-3 gene in cells of macrophage/microglial origin.

Materials and Methods

Recombinant proteins and reagents

Escherichia coli LPS (L5024) from E. coli 0127:B8, purified by ion-exchange chromatography (total impurities of <1% protein and <1% RNA), was purchased from Sigma-Aldrich. Recombinant murine M-CSF and IL-10 were from R&D Systems. Abs against phospho-STAT-1 Tyr701, phospho-STAT-3 Tyr705, phospho-ERK1/2 Thr202/Tyr204, phospho-JNK Thr183/Tyr185, and phospho-p38 MAPK Thr180/Tyr182 were purchased from Cell Signaling Technology. Abs against STAT-3, CREB-binding protein (CBP), p300, NF-κB p65, c-Jun, c-Fos STAT-1α, ERK1/2, JNK, p38, and actin were from Santa Cruz Biotechnology. Abs against acetylated histones H3 (AcH3) and H4 (AcH4) were from Upstate Biotechnology, whereas the Ab against RNA polymerase II (Pol II) was from Covance. The Ab against SOCS-3 was purchased as described previously (44). Abs against acetylated histones H3 (AcH3) and H4 (AcH4) were from Upstate Biotechnology, whereas the Ab against RNA polymerase II (Pol II) was from Covance. The Ab against SOCS-3 was purchased as described previously (44). Membranes were stripped at 50°C in buffer containing 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) with occasional shaking and reprobed for total STAT-1, STAT-3, ERK1/2, JNK, p38, or actin. SOCS-3 protein expression was analyzed using electrophoresis on 15% SDS-polyacrylamide gels and polyvinylidene difluoride membranes as described previously (35).

SOCS-3 promoter constructs, transient transfection, and luciferase assays

The 1619-bp (−1492 to +127 bp) murine SOCS-3 promoter was amplified from genomic DNA using the forward primer 5′-CCAGGCTACTCTGTAATGTCATGAACA-3′ and the reverse primer 5′-GCGAATCCGACGAGATAGCTGTTAGGAGTTCTTGTTGTA-3′ cloned into the pGL3 Basic vector. The SOCS-3 promoter sequence was confirmed by automatic sequencing (35). Serial deletion mutants were synthesized using PCR by Phusion polymerase to delete regulatory elements in the SOCS-3 promoter and put in the TA clone PCR2.1. The potential regulatory elements in the SOCS-3 promoter are three AP-1 sites, two GATA-1 sites, one NF-κB site, and two IFN-γ activation sequence (GAS) elements (see Fig. 2). They are named as follows: Δ1, which lacks the distal AP-1 site and the C/EBP site; Δ2, which lacks the two GATA-1 sites; Δ3, which lacks the medial AP-1 site; Δ4, which lacks the NF-κB element; Δ5, which lacks the two Sp1 elements; Δ6, which lacks the proximal AP-1 element; Δ7, which lacks the distal GAS element; and Δ8, which lacks the distal and proximal GAS elements (see Fig. 2). The deletion mutants were inserted into the pGL3-Basic vector, which contains the gene for luciferase as a reporter, by using the XbaI/BamHI restriction site. The site-directed mutation constructs were generated on the pGL3Δ5 (−120 bp) plasmid backbone using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions and were confirmed by sequencing. The SOCS-3 promoter constructs (0.2 μg) were transiently transfected into 5 × 10⁵ RAW264.7 cells in 6-well plates using the Lipofectamine Plus method as described previously (41). Transfected cells were treated with medium or LPS for 12 h, and the luciferase activity of each sample was normalized to the total protein content of each well. The luciferase activity from the untested sample was arbitrarily set at 1 for the calculation of fold induction.

ELISA

Supernatants were collected from unstimulated or LPS-stimulated RAW264.7 cells and assayed by ELISA for secretion of IL-10 (PBL Biomedical Laboratories). IL-10 levels were normalized to total protein levels.

RNA interference

RNA interference was done following a protocol provided by Dharmacon with modifications as previously described (45). DharmaFECT 1 small interfering RNA (siRNA) transfection reagent, SMARTpool siRNAs specific for murine STAT-3, and siCONTROL nontargeting siRNAs were purchased from Dharmacon. RAW264.7 cells (5 × 10⁵) in 6-well plates were transfected with 100 nM siCONTROL or STAT-3 specific siRNAs using the DharmaFECT 1 reagent following the manufacturer’s recommendations. After 48 h of transfection, 30 μg of cell lysate was subjected to immunoblotting for STAT-3 expression and STAT-3 phosphorylation as described above.
LPS induces SOCS-3 gene expression in macrophages and microglia. A, RAW264.7 cells were treated with medium (UN) or LPS (10 ng/ml) for up to 36 h and then total RNA was isolated and analyzed by RPA for SOCS-3 and GAPDH mRNA. The basal level of the untreated sample was set at 1.0, and fold induction upon LPS treatment was compared with that value. The experiment shown is representative of at least three experiments. B, RAW264.7 cells were treated with LPS for up to 24 h and then cell lysates were subjected to immunoblotting with anti-SOCS-3 Ab and stripped and reprobed with anti-actin Ab as a loading control. The experiment shown is representative of at least three experiments. C, BV2 microglial cells were treated with medium (UN) or LPS for up to 24 h and then total RNA was isolated and analyzed by RPA for SOCS-3 and GAPDH mRNA expression. The basal level of the untreated sample was set at 1.0, and fold induction upon LPS treatment was compared with that value. The experiment shown is representative of at least three experiments. D, Primary murine microglia were treated with medium (UN) or LPS for up to 12 h and then total RNA was isolated and analyzed by RPA for SOCS-3 and GAPDH mRNA expression. The basal level of the untreated sample was set at 1.0, and fold induction upon LPS treatment was compared with that value. The experiment shown is representative of at least three experiments.

Chromatin immunoprecipitation (ChIP) assays

ChIP analysis was done following a protocol provided by Upstate Biotechnology with modifications as described previously (34, 44). RAW264.7 cells were incubated with medium or LPS for up to 4 h, fixed with 1% formaldehyde for 15 min at room temperature, and nuclei were isolated. Chromatin was sheared by sonication and samples were precleared for 2 h at 4°C with salmon sperm DNA-saturated protein A/G-Sepharose. Chromatin solutions were precipitated overnight at 4°C with 5 μg of Abs or isotype-matched control IgG. Input chromatin and immunoprecipitated chromatin were incubated at 65°C overnight to reverse cross-links. After proteinase K digestion, DNA was extracted with the Qiagen Miniprep kit. Purified DNA was analyzed by PCR with Taq polymerase. The primer pair 5′-GCTTTGTCTCCTCCTCGGTAGG-3′ and 5′-AGT GTAGAGTCAGAGTTAGAGCC-3′ was used to amplify a 230-bp region of the SOCS-3 promoter containing the distal AP-1 element and the two GAS elements. Densitometry was used to quantify the PCR results, and all results were normalized by the respective input values.

Statistical analysis

All experiments were repeated a minimum of three times. Levels of significance for comparison between samples were determined by the Student’s t test distribution. A value of p < 0.05 was considered to be statistically significant.

Results

LPS induced SOCS-3 expression in macrophages and microglia

We have recently demonstrated that LPS-induced SOCS-3 plays an important role in the negative regulation of CD40 gene expression in macrophages and microglia (35). As such, we have undertaken an analysis of how LPS regulates SOCS-3 gene expression in these cells. SOCS-3 mRNA expression was slightly induced at 0.5 h after the addition of LPS, peaked at 8 h (15.9-fold induction), persisted up to 24 h, and diminished at 36 h in RAW264.7 murine macrophage cells (Fig. 1A). SOCS-3 protein expression was detectable 1 h after LPS stimulation, peaked at 8 h, returned to basal levels at 24 h, and came back at 36 h as assessed by immunoblotting (Fig. 1B). In BV2 cells, a murine microglial cell line, LPS-induced SOCS-3 mRNA expression was detected at 0.5 h, peaked at 8 h (~12.9-fold induction), and expression persisted up to 16–24 h, albeit at lower levels (Fig. 1C). Primary murine microglia were treated with LPS for up to 12 h, and SOCS-3 mRNA was strongly induced at all of the time points tested (Fig. 1D). These results collectively demonstrate that LPS induces SOCS-3 mRNA and protein expression in a time-dependent manner in murine macrophage and microglial cell lines and in primary microglia.

LPS-induced SOCS-3 promoter activity required AP-1 and GAS elements

Within the murine SOCS-3 promoter three potential AP-1 sites, one NF-κB, and two GAS-like elements were found by the TFSEARCH program (Fig. 2) (46). To ascertain the functional roles of these AP-1, NF-κB, and GAS cis-regulatory elements as well as other potential regulatory elements, deletion constructs were generated from the 5′ end of the SOCS-3 promoter as described in Materials and Methods. The WT SOCS-3 promoter and various deletion constructs were transiently transfected into RAW264.7 cells, which were treated with medium or LPS for 12 h and then assayed for luciferase activity. LPS stimulation caused a 6.9-fold induction of WT SOCS-3 promoter activity. Deletion of the distal
FIGURE 2. LPS induces activation of the murine SOCS-3 promoter. A, RAW264.7 cells were transiently transfected with 0.2 μg of the indicated constructs, allowed to recover for 4 h, treated with medium or LPS (10 ng/ml) for 12 h, and then analyzed for luciferase activity. Values were normalized to total protein levels and fold induction was calculated by dividing the LPS treatment values by the untreated values. Data are presented as the mean ± SD of three experiments. B, RAW264.7 cells were transiently transfected with 0.2 μg of the Δ1, the NF-κB site (Δ4), and two Sp1 elements (Δ5) had no significant effect on LPS-induced SOCS-3 promoter activity compared with the Δ1 construct. In construct Δ6, deletion of the proximal AP-1 element reduced LPS-induced SOCS-3 promoter activity by ~51% compared with Δ1. Deletion of the distal GAS element (GAS no.1) (Δ7) further reduced LPS-induced SOCS-3 promoter activity compared with Δ1. In construct Δ8, deletion of both the GAS elements, GAS no.1 and GAS no.2, abrogated LPS-induced SOCS-3 promoter activity. These data indicate that the proximal AP-1 and the two GAS elements are important for LPS-induced SOCS-3 promoter activity.

To better define the contribution of the two GAS elements, site-directed mutagenesis was used to individually mutate each GAS element (Fig. 2B). Mutation of the distal GAS no.1 element reduced LPS-induced SOCS-3 promoter activity by ~57%, whereas mutation of the proximal GAS no.2 element abrogated LPS-induced SOCS-3 promoter activity (Fig. 2B). These results suggest that the proximal GAS no.2 element has a pivotal role in LPS-induced SOCS-3 promoter activity while the distal GAS no.1 element is also important in this response.

Deletion of the NF-κB element in the SOCS-3 promoter (Δ4) did not significantly influence LPS-induced SOCS-3 promoter activity (Fig. 2). To further assess the potential involvement of NF-κB, dominant negative (DN) expression constructs of IκB kinase (IKK)-α or IKK-β were used that contain substitutions of alanine for an essential lysine in the ATP-binding site, rendering the proteins catalytically inactive (47). We have previously used these constructs to demonstrate the involvement of the NF-κB pathway in LPS-induced CD40 expression (34). DN IKK-α and IKK-β constructs were cotransfected with the WT SOCS-3 promoter construct into RAW264.7 cells and then treated with medium or LPS. Cotransfection with either construct did not affect LPS-induced SOCS-3 promoter activity (data not shown), indicating that the NF-κB signaling pathway is not involved in this response.

STAT-3, but not STAT-1α, was critical for LPS-induced SOCS-3 gene expression

LPS induces activation of both STAT-1 and STAT-3 in macrophages/microglia as assessed by phosphorylation status (34, 35). To determine whether STAT-1 was involved in LPS-induced SOCS-3 expression, primary microglia and BMDM from STAT-1α-deficient mice were examined. The absence of STAT-1α led to an enhancement of LPS-induced SOCS-3 mRNA, particularly at the later time points (Fig. 3, A and B). These experiments demonstrate that LPS induction of SOCS-3 expression occurred in a STAT-1α-independent manner and that STAT-1α may function as a negative regulator of SOCS-3. We next examined LPS activation of STAT-1α and STAT-3 in WT and STAT-1α-deficient BMDM. LPS induced STAT-1α and STAT-3 tyrosine phosphorylation in a time-dependent manner in WT BMDM (Fig. 3C, lanes 3–5). Interestingly, LPS-induced STAT-3 phosphorylation was prolonged in STAT-1α-deficient BMDM compared with WT cells (Fig. 3C, compare lanes 5 and 10), suggesting that enhanced STAT-3 signaling in STAT-1α deficient cells may contribute to SOCS-3 expression.

To directly test whether STAT-3 was required for LPS induction of SOCS-3, STAT-3 siRNA was used to knock down STAT-3 expression. RAW264.7 cells were incubated with STAT-3 siRNA (RAW-STAT-3i) (100 nM) or siRNA control (100 nM) for 48 h and then STAT-3 protein expression was assessed. Constitutive STAT-3 protein levels were inhibited (~90%) using SMARTpool siRNA (Dharmacon) specific for murine STAT-3 (Fig. 4A, compare lanes 1 and 3). LPS induced a modest increase in STAT-3 expression (Fig. 4A, lane 2), which was inhibited (~86%) in...
RAW-STAT-3i cells (lane 4). LPS-induced STAT-3 phosphorylation was inhibited (~78%) in RAW-STAT-3i cells compared with siRNA control cells (Fig. 4A, lane 4 vs lane 2). RAW264.7 cells were incubated with STAT-3 siRNA or control siRNA for 48 h and then LPS-induced SOCS-3 mRNA expression was examined. SOCS-3 expression was partially inhibited in RAW-STAT-3i cells (~53%) compared with siRNA-control cells (Fig. 4B, compare lanes 2 and 4). LPS-induced SOCS-3 promoter activity was significantly inhibited by ~75% in RAW-STAT-3i cells (Fig. 4C). To further investigate the role of STAT-3, a constitutively active STAT-3 (STAT-3C) construct was used (39). RAW264.7 cells were transiently transfected with increasing concentrations of STAT-3C and the WT SOCS-3 promoter. As shown in Fig. 4D, the inclusion of STAT-3C activated the SOCS-3 promoter activity in a dose-dependent manner. These results collectively indicate that STAT-3 activation plays a critical role in SOCS-3 gene expression.

LPS-induced endogenous IL-10 is important for SOCS-3 gene expression

The results from Fig. 1 indicate that LPS activation of SOCS-3 mRNA is sustained out to 24 h, suggesting that synthesis of an intermediate protein(s) may be important for SOCS-3 expression, particularly at later times. To determine whether LPS-induced SOCS-3 gene expression required de novo protein synthesis, experiments were conducted using the protein synthesis inhibitor cycloheximide (CHX). RAW264.7 cells were incubated with medium, LPS, CHX (5 μg/ml), or CHX plus LPS for 8 h and then RNA was extracted and analyzed by RNase protection assay (RPA). CHX alone had no effect on SOCS-3 mRNA expression.
while the inclusion of CHX reduced LPS-induced SOCS-3 mRNA expression by ∼76% (lane 4). The inhibitory effect of CHX was not observed at earlier time points (1–4 h) (data not shown). Thus, for optimal induction of SOCS-3 mRNA expression by LPS at later time points, de novo protein synthesis is required.
FIGURE 6. LPS activation of the ERK, JNK, and p38 MAPK pathways are involved in SOCS-3 gene expression. A. RAW264.7 cells were treated with medium (UN), LPS, LPS plus the ERK inhibitor U0126 (10 μM), LPS plus the JNK inhibitor II (10 μM), or LPS plus the p38 inhibitor SB230580 (10 μM) for 4 h and then cell lysates were subjected to immunoblotting with anti-phospho-ERK1/2 (p-ERK 1/2), anti-phospho-JNK (p-JNK), or anti-phospho-p38 (p-p38) and then stripped and reprobed with anti-ERK1/2, anti-JNK, or anti-actin Abs as loading controls. The experiment shown is representative of at least three experiments.

B. RAW264.7 cells were transiently transfected with the murine SOCS-3 promoter construct, treated with medium (UN), LPS, LPS plus ERK inhibitor U0126, LPS plus JNK inhibitor II (JNKi II), or LPS plus p38 inhibitor SB230580 for 4 h and then cell lysates were subjected to immunoblotting with anti-phosphotyrosine (P-Tyr)-STAT-3 and stripped and reprobed with anti-STAT-3 and anti-actin Abs as loading controls. The experiment shown is representative of at least three experiments.

C. LPS activation of the ERK, JNK, and p38 MAPK pathways. LPS (100 ng/ml) was added to RAW264.7 cells for 8 h and then RNA was analyzed by RPA for SOCS-3 and GAPDH mRNA. The basal level of the untreated sample was set at 1.0 and fold induction upon treatment was compared with that value. The experiment shown is representative of at least three experiments.

D. RAW264.7 cells were treated with medium (UN), LPS, LPS plus ERK inhibitor U0126 (10 μM), LPS plus JNK inhibitor II (JNKi II) (10 μM), or LPS plus p38 inhibitor SB230580 (10 μM) for up to 4 h and then cell lysates were subjected to immunoblotting with anti-phospho-ERK1/2 (p-ERK 1/2), anti-phospho-JNK (p-JNK), or anti-phospho-p38 (p-p38) and then stripped and reprobed with anti-STAT-3 and anti-actin Abs as loading controls. The experiment shown is representative of at least three experiments.

E. RAW264.7 cells were transiently transfected with the murine SOCS-3 promoter construct, treated with medium (UN), LPS, LPS plus ERK inhibitor U0126, LPS plus JNK inhibitor II (JNKi II), or LPS plus p38 inhibitor SB230580 for 8 h and then RNA was analyzed by RPA for SOCS-3 and GAPDH mRNA. The basal level of the untreated sample was set at 1.0 and fold induction upon treatment was compared with that value. The experiment shown is representative of at least three experiments.

ERK1/2, JNK, and p38 MAPK pathways were involved in LPS-induced SOCS-3 gene expression

ERK1/2, JNK, and p38 MAPK pathways were involved in LPS-induced SOCS-3 gene expression. The basal level of the untreated sample was set at 1.0 and fold induction upon treatment was compared with that value. The experiment shown is representative of at least three experiments. D. RAW264.7 cells were treated with medium (UN), LPS, LPS plus ERK inhibitor U0126 (10 μM), LPS plus JNK inhibitor II (JNKi II) (10 μM), or LPS plus p38 inhibitor SB230580 (10 μM) for 12 h and then analyzed for luciferase activity. Data are presented as mean ± SD of three experiments. *p ≤ 0.05; **p ≤ 0.001 compared with LPS alone.

IL-10 induced SOCS-3 gene expression in RAW264.7 cells that depended on the activation of STAT-3. To determine whether LPS-induced IL-10 production is involved in SOCS-3 gene expression, IL-10 mRNA expression and protein expression were analyzed. LPS-induced IL-10 mRNA expression was detected 2 h after LPS treatment, peaked at 4 h, and then decreased at 16 h in RAW264.7 cells (Fig. 5B). IL-10 protein expression was detected 4 h after LPS treatment, peaked between 8 and 12 h, and then declined slightly at 24 h (Fig. 5C). These experiments were repeated in BMDM and microglial cells and similar results were obtained, although the kinetics of IL-10 mRNA expression was more rapid in these cells (i.e., diminished at 8–12 h) (data not shown).

To examine whether LPS-induced endogenous IL-10 was involved in subsequent SOCS-3 gene expression, BMDM from IL-10-deficient and WT mice were analyzed. The results shown in Fig. 5D demonstrate that IL-10 deficiency does not affect SOCS-3 expression at early time points (1, 2, and 4 h); however, at later time points (8 and 12 h) LPS-induced SOCS-3 expression was decreased (~60%) in BMDM from IL-10-deficient mice compared with WT mice. This is in agreement with the time point of optimal IL-10 protein expression. To determine whether exogenous IL-10 could rescue LPS-induced SOCS-3 expression, BMDM from IL-10-deficient and WT mice were treated with IL-10 (Fig. 5E). IL-10 alone induced SOCS-3 gene expression in BMDM from both WT and IL-10-deficient mice, but the extent of SOCS-3 expression was more pronounced in WT BMDM (Fig. 5E, lane 3) compared with IL-10-deficient BMDM (lane 6). Next, the levels of phosphorylated STAT-3 were tested in these cells. LPS-induced STAT-3 activation was absent in IL-10-deficient BMDM compared with WT BMDM (Fig. 5F, lanes 2 and 5), and the addition of IL-10 led to STAT-3 activation in both WT and IL-10-deficient BMDM (lanes 3 and 6), with the response in WT BMDM being stronger. Taken together, these results indicate that LPS-induced expression of endogenous IL-10 contributes to SOCS-3 gene expression at later time points, likely through the activation of STAT-3.

ERK1/2, JNK, and p38 MAPK pathways were involved in LPS-induced SOCS-3 gene expression

In the absence of STAT-3, SOCS-3 expression was not totally inhibited, suggesting that other pathways are involved in this response. To investigate this, the MAPK pathways were evaluated.

or LPS plus the p38 inhibitor SB230580 (10 μM) for 4 h and then cell lysates were subjected to immunoblotting with anti-phosphotyrosine (P-Tyr)-STAT-3 and stripped and reprobed with anti-STAT-3 and anti-actin Abs as loading controls. The experiment shown is representative of at least three experiments. C. RAW264.7 cells were treated with medium (UN), LPS, LPS plus ERK inhibitor U0126 (10 μM), LPS plus JNK inhibitor II (10 μM), or LPS plus p38 inhibitor SB230580 (10 μM) for 8 h and then RNA was analyzed by RPA for SOCS-3 and GAPDH mRNA. The basal level of the untreated sample was set at 1.0 and fold induction upon treatment was compared with that value. The experiment shown is representative of at least three experiments. D. RAW264.7 cells were transiently transfected with the murine SOCS-3 promoter construct, treated with medium (UN), LPS, LPS plus ERK inhibitor U0126, LPS plus JNK inhibitor II (JNKi II), or LPS plus p38 inhibitor SB230580 for 8 h and then analyzed for luciferase activity. Data are presented as mean ± SD of three experiments. *p ≤ 0.05; **p ≤ 0.001 compared with LPS alone.

E. RAW264.7 cells were transiently transfected with the murine SOCS-3 promoter construct plus pcDNA3 or the MEK1-DN construct (100–500 ng), treated with medium or LPS for 12 h, and analyzed for luciferase activity. Data are presented as mean ± SD of three experiments. **p ≤ 0.001 compared with pcDNA3.
LPS treatment resulted in the phosphorylation of ERK1 and ERK2 (ERK1/2), JNK and p38 MAPK in a time-dependent manner (Fig. 6A, lanes 1–6). To determine the role of these MAPK pathways in LPS-induced SOCS-3 expression, specific pharmacological inhibitors for ERK1/2, JNK, and the p38 pathways were used. As shown in Fig. 6A, ERK1/2 activation was blocked by the MEK-1 inhibitor U0126, JNK activation was blocked by the JNK inhibitor II, and p38 MAPK activation was inhibited by SB230580, respectively (lanes 8–12). The effect of the MAPK inhibitors on LPS-induced STAT-3 tyrosine phosphorylation was also tested to determine specificity (Fig. 6B). The inclusion of U0126 (Fig. 6B, lane 3), JNK inhibitor II (lane 4), or SB230580 (lane 5) did not inhibit LPS-induced STAT-3 phosphorylation. In fact, U0126 modestly enhanced LPS-induced STAT-3 phosphorylation.

U0126 (Fig. 6C, lanes 3–5), JNK inhibitor II (lanes 6–8), and SB230580 (lanes 9–11) inhibited LPS-induced SOCS-3 mRNA expression in a dose-dependent manner to varying degrees and also inhibited LPS-induced SOCS-3 promoter activity in a dose-dependent manner (Fig. 6D). Inhibition of ERK1/2 by U0126 resulted in the most pronounced inhibition of LPS-induced SOCS-3 mRNA expression and promoter activity compared with the JNK and p38 inhibitors (Fig. 6, C and D), suggesting that activation of the ERK1/2 pathway is an important contributor to LPS-induced SOCS-3 gene expression. To further investigate the role of ERK1/2 in LPS-induced SOCS-3 expression, a DN MEK-1(K/A) construct was used. The results demonstrated that inclusion of MEK-1(K/A) inhibited LPS-induced SOCS-3 promoter activity (Fig. 6E). Thus, activation of the MEK-1/ERK pathway was involved in LPS-induced SOCS-3 gene expression, with lesser contributions from the JNK and p38 pathways.

Recruitment of STAT-3, c-Jun, c-Fos, RNA Pol II, and the coactivators CBP and p300 to the SOCS-3 promoter and modification of histone H3 and H4 acetylation in response to LPS

We have shown that STAT-3 and MEK-1/ERK1/2 activation are involved in LPS-induced SOCS-3 gene transcription. However, it is still unknown whether STAT-3 and the AP-1 transcription factors, which are downstream targets of ERK1/2, are recruited to the endogenous SOCS-3 promoter and how this is coupled to SOCS-3 gene transcription. To monitor transcription factor binding to the endogenous SOCS-3 promoter, RAW264.7 cells were incubated in the absence or presence of LPS for up to 4 h and ChIP assays were performed using Abs against STAT-3, the AP-1 subunits c-Jun and c-Fos, or normal rabbit IgG (as a negative control). PCR analysis of the positive control (input) indicated that the soluble chromatin samples obtained from each time point had equal amounts of chromatin fragments containing the SOCS-3 promoter (Fig. 7A). STAT-3 was not associated with the SOCS-3 promoter in untreated cells, and a small increase in STAT-3 binding was observed at 1 h after LPS treatment (Fig. 7A). Optimal recruitment of STAT-3 to the SOCS-3 promoter was observed between 2 and 4 h of LPS treatment. c-Jun was weakly associated with the SOCS-3 promoter in untreated RAW264.7 cells. The peak of c-Jun recruitment to LPS was at 30 min, although binding was still observed at 1–2 h (Fig. 7A). c-Fos was also weakly associated with the SOCS-3 promoter in untreated RAW264.7 cells. Increased binding of c-Fos peaked at 15 min after LPS treatment; however, the binding of c-Fos was still strong from 30 min to 2 h after LPS treatment and then diminished at the 4-h time point (Fig. 7A). ChIP analysis with anti-STAT-1 and NF-kB p65 Abs demonstrated that neither transcription factor was recruited to the SOCS-3 promoter after LPS stimulation (data not shown). These results indicate that LPS stimulation induces recruitment of c-Jun, c-Fos, and STAT-3 to the SOCS-3 promoter in a temporal manner, with c-Jun and c-Fos recruited early (15 min to 1 h), followed by STAT-3 at 2–4 h.

Covalent histone modifications have functional roles in gene transcription (48). The N-terminal tails of core histones are subject to modifications such as acetylation, methylation, phosphorylation, and ubiquitination (49). To study histone modifications during LPS-induced SOCS-3 gene transcription, ChIP assays were performed. The acetylation of histone H3 was increased from 15 min to 1 h following LPS treatment, peaked at 2 h, and then diminished at 4 h after treatment (Fig. 7B). Enhanced acetylation of histone H4 peaked at 15 min following LPS treatment and then diminished over time (Fig. 7B). Histone acetylation of lysine residues requires the activities of histone acetyltransferases such as CBP/p300, which are important in relaxing the compact structure of nucleosomes (50). CBP and p300 were weakly associated with the
SOCS-3 promoter in untreated RAW264.7 cells, and LPS stimulation promoted recruitment of CBP from 15 min to 4 h and recruitment of p300 from 15 min to 2 h after treatment (Fig. 7B). ChIP assays were also performed for the recruitment of RNA Pol II to the SOCS-3 promoter. Pol II was not associated with the SOCS-3 promoter in untreated RAW264.7 cells; however, recruitment was observed at 30 min after LPS addition, diminished slightly at 1 h, peaked at 2 h, and then diminished again at 4 h (Fig. 7B). These results suggest that LPS induces histone H3 and H4 modifications on the SOCS-3 promoter, concurrent with CBP, p300, and RNA Pol II recruitment to initiate transcription of the SOCS-3 gene.

**Discussion**

A precise regulation of both the magnitude and duration of cytokine signaling is essential for orchestration of numerous biological processes, including innate and adaptive immune responses. The SOCS-3 protein has an important role in mitigating pathogenic effects of cytokine-induced immune and inflammatory responses. Using LPS as a surrogate ligand for cytokines and other stimuli, we provide new information on the molecular mechanisms underlying SOCS-3 expression in macrophages and microglia. LPS treatment of cells promotes rapid activation of MAPK signaling pathways (ERK1/2, p38, and JNK), the induction of IL-10, and the subsequent activation of STAT-3, events that collectively contribute to optimal SOCS-3 gene expression. Furthermore, we demonstrate that the transcription factors c-Jun, c-Fos, and STAT-3 are recruited to the SOCS-3 promoter in vivo in a stepwise manner upon LPS stimulation. The coactivators CBP and p300 were also recruited to the SOCS-3 promoter in a temporal fashion that coincides with permissive histone modifications (H3 and H4 acetylation) and activation of the SOCS-3 gene. Thus, LPS activation of intracellular signaling cascades, histone modifications, and temporal recruitment of transcription regulators is critical to regulate SOCS-3 gene expression (Fig. 8).

The SOCS-3 promoter contains numerous potential regulatory elements, including AP-1, C/EBP\(\beta\), NF-\(\kappa\)B, Sp1, and GAS-like binding sites. Our results implicate the proximal AP-1 element and the two GAS-like elements in SOCS-3 gene transcription by LPS. Our data indicate that there are stimulus-specific differences in the use of regulatory elements and transcription factors involved in SOCS-3 expression. Gatto et al. (51) demonstrated that IFN-\(\gamma\) induction of SOCS-3 involved STAT-1 binding to the most proximal GAS-like element in the SOCS-3 promoter. Furthermore, SOCS-3 expression was independent of STAT-3, as IFN-\(\gamma\) strongly induced SOCS-3 in STAT-3-deficient cells. LIF induction of SOCS-3 involves both STAT-1 and STAT-3, as STAT-1 homodimers, STAT-3 homodimers, and STAT-1/STAT-3 heterodimers all bind to the proximal GAS-like element in the SOCS-3 promoter (16). Our findings indicate that for LPS induction of SOCS-3, both GAS elements are involved and that STAT-3, but not STAT-1, is critical in this response. These results are supported by the fact that LPS induced SOCS-3 expression in STAT-1-deficient macrophages and microglia and that STAT-1 is not detected at the endogenous SOCS-3 promoter. Our findings are in agreement with those of Ramana et al. (52) who demonstrated that IFN-\(\gamma\) induction of SOCS-3 is evident in STAT-1-deficient mouse fibroblasts and is associated with more pronounced STAT-3 activation. In our study, involvement of STAT-3 was documented by the use of STAT-3 siRNA in which the inhibition of STAT-3 expression partially inhibited SOCS-3 induction in response to LPS. These results collectively indicate that STAT-3, but not STAT-1, is critical for LPS stimulation of SOCS-3 gene expression in macrophages/microglia.

**FIGURE 8.** Proposed model for LPS-induced SOCS-3 gene expression. LPS activates the MAPK pathways ERK1/2, JNK, and p38, which lead to nuclear translocation and binding of c-Jun and c-Fos to the SOCS-3 promoter. Concurrently, LPS induces IL-10 expression, which leads to the subsequent activation of STAT-3, translocation into the nucleus, and binding to the SOCS-3 promoter with delayed kinetics compared with that of c-Jun and c-Fos. LPS treatment also leads to activation of the coactivators CBP and p300, modifications in H3 and H4 acetylation (Ac), and recruitment of RNA Pol II. The sequential recruitment of transcription factors, coactivators, and RNA Pol II to the SOCS-3 promoter, in conjunction with permissive histone modifications, results in transcriptional activation of the SOCS-3 gene. See **Discussion** for details.

The MAPK pathway has been implicated in SOCS-3 expression induced by CpG-DNA, LPS, TNF-\(\alpha\), IL-4, IL-6, and *Mycobacterium avium* (15, 37, 38, 53, 54), but the molecular basis underlying these responses is not well understood. Our results implicate the MEK-1/ERK1/2, p38 MAPK, and JNK pathways in LPS induction of SOCS-3 expression because pharmacological inhibitors of the three pathways inhibited, to varying degrees, SOCS-3 expression. Furthermore, a DN MEK-1 construct inhibited LPS-induced SOCS-3 promoter activity. More importantly, we demonstrate for the first time recruitment of the transcription factors c-Jun and c-Fos to the endogenous SOCS-3 promoter in response to LPS. This recruitment occurs with rapid kinetics (15–60 min), then diminishes over time. Thus, we believe that c-Jun and c-Fos are important for the initial phase of LPS induction of SOCS-3 gene expression.

We and others have demonstrated that IL-10 induces SOCS-3 expression, which may contribute to the anti-inflammatory activity of IL-10 (12, 35, 55, 56). IL-10 is a potent activator of STAT-3 (55, 57–59), and we have previously shown that STAT-3 activation is critical for IL-10-induced SOCS-3 expression (35). In this study, use of IL-10-deficient macrophages revealed that IL-10 expression is necessary for optimal LPS induction of SOCS-3, especially at later time points. This finding is consistent with the observation that LPS induction of SOCS-3 is inhibited by CHX at later time points (8 h), which coincides with LPS induction of IL-10 expression. Thus, our findings indicate that IL-10 is an intermediary cytokine involved in SOCS-3 expression induced by LPS and may account for the sustained expression of SOCS-3 mRNA. Staples et al. (58) have recently shown that IL-10 induces its own expression in monocyte-derived macrophages via activation of STAT-3. This positive autocrine feedback loop may allow IL-10 to enhance its effect on SOCS-3 gene expression. Our results also demonstrate that a neutralizing Ab to TNF-\(\alpha\) has no effect on
LPS-induced SOCS-3 mRNA expression (data not shown), excluding the involvement of TNF-α in mediating SOCS-3 expression. The need for protein synthesis for SOCS-3 expression is not required in all contexts. In the Bac1.2F5 macrophage cell line, LPS induction of SOCS-3 mRNA expression was maximal at 4 h and was unaffected by cycloheximide treatment (36). SOCS-3 mRNA induction by Borrelia burgdorferi in J774 macrophages is independent of ongoing protein synthesis and does not require endogenous production of IL-10 (60).

The function of SOCS proteins in cells of the CNS is complex. In a spinal cord injury model, conditional deletion of SOCS-3 in astrocytes led to the quicker formation of glial scars, thus preventing excessive infiltration of inflammatory cells, sparing myelin and oligodendrocytes, and ultimately improving functional recovery (61). In contrast, conditional deletion of STAT-3 in astrocytes inhibited astrocyte migration and subsequent glial scar formation and thus delayed functional recovery. These findings indicate that SOCS-3 represses the ability of reactive astrocytes to promote healing after spinal cord injury by blocking STAT-3 activation.

Conditional deletion of SOCS-3 in oligodendrocytes enhanced the protective effect of LIF on cuprizone-induced demyelination (62), demonstrating that SOCS-3 expression in oligodendrocytes attenuates the beneficial effects of LIF in limiting demyelination. In contrast, targeted expression of SOCS-1 in oligodendrocytes prevents them against the injurious effects of IFN-γ (63). Our findings in macrophages/microglia indicate that both SOCS-1 and SOCS-3 attenuate inflammatory events in these cells (35, 45, 64). Thus, depending on the CNS cell type, SOCS-3 expression exerts beneficial or detrimental effects.

It is also important to consider the function of SOCS-3 in dendritic cells (DCs) and T cells that migrate into the CNS under neuropathological conditions. Li et al., (65) demonstrated that SOCS-3-transduced DCs promoted Th2 polarization of naive CD4+ T cells and attenuated disease severity in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. SOCS-3-deficient CD4+ T cells produce more TGF-β and IL-10 but less IL-4 than control cells, suggesting preferential Th3-like differentiation (66). More recently, SOCS-3 was shown to regulate Th17 cell differentiation by inhibiting IL-23-mediated STAT-3 signaling, indicating that SOCS-3 has an important function in constraining IL-17 production (67). Thus, the expression of SOCS-3 in DCs and T cells may skew the CNS microenvironment toward a protective Th2 and/or Th3 phenotype and prevent Th17 cell development.

The induction and regulation of SOCS-3 expression in macrophages/microglia, oligodendrocytes, astrocytes, DCs, T cells, and neurons will ultimately dictate the extent of negative regulation of immune and inflammatory responses within the CNS, which is relevant to the pathogenesis of autoimmune and neurodegenerative diseases. A detailed understanding of the transcriptional regulation of SOCS-3 gene expression, which is both stimulus and cell type specific, will provide the foundation for how SOCS-3 expression can be harnessed to regulate complicated cytokine signaling networks in the brain and other organ sites.

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Disclosures
The authors have no financial conflict of interest.

References


