Suppressor of cytokine signaling (SOCS) proteins constitute a class of negative regulators for Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways. These intracellular proteins are induced by cytokine signaling, but they can also be induced by stimulation of Toll-like receptors (TLR). It has even been suggested that SOCS proteins are important negative regulators of TLR signaling. Here we have elucidated the nature of the regulatory role of SOCS in TLR signaling. Induction of SOCS-3 and cytokine-inducible Src homology 2-containing protein (CIS) by TLR stimulation was strictly dependent on MyD88 but showed differing needs in case of SOCS-1. However, induction of SOCS proteins by TLR ligands was independent of type I interferon. In macrophages overexpressing SOCS, we were not able to observe an inhibitory effect of SOCS-1, SOCS-2, SOCS-3, or CIS on prototypical TLR target genes such as tumor necrosis factor-α. However, we found that TLR-2, TLR-3, TLR-4, and TLR-9 stimulation induced interferon-β (IFN-β), which is able to exert auto- and paracrine signaling, leading to the activation of secondary genes like IP-10. SOCS-1 and, to a lesser extent, SOCS-3 and CIS were able to inhibit this indirect signaling pathway following TLR stimulation, whereas neither MAP kinase nor NFκB signaling were affected. However, STAT-1 tyrosine phosphorylation following TLR triggering was severely impaired by SOCS-1 overexpression. Thus, our data suggest that SOCS proteins induced by TLR stimulation limit the extent of TLR signaling by inhibiting type I IFN signaling but not the main NFκB pathway.

Suppressor of cytokine signaling (SOCS)1 proteins have been identified as negative feedback inhibitors for various cytokines.

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1 The abbreviations used are: SOCS, suppressor of cytokine signaling; SH2, Src homology 2; CIS, cytokine-inducible SH2-containing protein; GM-CSF, granulocyte macrophage-colony-stimulating factor; IFN, interferon; IFNAR, interferon-α receptor; IRAK, interleukin-1 receptor-associated kinase; JAK, Janus kinase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; Mal, MyD88-adaptor like; PBS, phosphate-buffered saline; STAT, signal transducer and activator of transcription; TICAM, TLR-containing adaptor molecule; TRIF, Toll-interleukin-1 receptor domain; TRAM, TRIF-related adaptor molecule; TRAF, TNF receptor-associated factor; TRIF, TLR-containing adaptor-inducing interferon-β; MAP, mitogen-activated protein; iNOS, inducible nitric-oxide synthase; ODN, oligonucleotide; FCS, fetal calf serum; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; PE, phycoerythrin; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; Ab, antibody.
SOCS Proteins Indirectly Inhibit TLR Signaling

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mutations in TLRs result in impaired defense against infections (22). Upon TLR stimulation, at least some of the receptors dimerize. In turn, adaptor molecules are recruited to the receptor complex via the TIR domain that is shared with the interleukin-1 receptor. Subsequently, further signaling molecules among which are IRAKs and TRAF-6 are recruited and activate MAP kinase signaling, as well as NFκB. MyD88 was the first adaptor molecule to be identified, and MyD88-deficient mice show a loss of proinflammatory cytokine induction upon TLR stimulation (23). However, some signaling events are independent of MyD88, and further adaptors have been identified. Thus, a complicated picture has emerged in which two main signaling streams can be observed (24). First, there are direct target genes, which are induced via MAP kinases and NFκB, including the prototypical proinflammatory cytokine TNF-α. Some of these genes, however, are induced upon intermediate activation of ILBc, as shown recently (25). This pathway is dependent on MyD88; however, in the case of TLR-2 and TLR-4, an additional adaptor Mal/TIRAP can be used (26, 27). Second, at least some TLRs activate IRF-3, resulting in IFN-β induction (24). In turn, IFN-β becomes secreted and can act in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes. IP-10 and iNOS are induced in an autocrine and paracrine manner to activate JAK/STAT signaling. This broadens the set of induced genes.

There is indirect evidence linking SOCS to TLR signaling, since it has been reported that SOCS-1-deficient mice exhibit increased LPS sensitivity, associated with elevated TNF-α secretion, and lack endotoxin tolerance (35, 36). These effects could be observed in macrophages and were also retained in SOCS-1/IFN-γ double knock-out mice, thus, ruling out the possibility of a pre-activation of macrophages by increased IFN-γ sensitivity. Direct effects of SOCS-1 on NFκB signaling have been proposed on the basis of transfection experiments. However, the precise mechanism and role of SOCS-1 for TLR signaling are still elusive.

Here we analyzed the MyD88 and paracrine type I IFN dependence of SOCS-1, SOCS-3, and CIS induction following TLR stimulation. Moreover, we made use of an overexpression system of SOCS in macrophages with careful control of expression levels. We were not able to observe inhibitory effects of SOCS on TLR-induced TNF-α and nitrite secretion. However, we found that all TLR ligands induced IFN-β and subsequent IFN-β-triggered STAT-1-dependent signaling. This secondary signaling pathway, as exemplified by IP-10 induction, was sensitive to inhibition by SOCS-1 and to a lesser extent by SOCS-3 and CIS. These data clearly show that the effects of SOCS-1 on TLR signal transduction are limited to the inhibition of secondary type I IFN-dependent amplification circuits.

**EXPERIMENTAL PROCEDURES**

*Reagents—*Completely phosphorothiolate-modified CpG-oligodeoxynucleotide (ODN) 1668 (TCC ATG ACG TTC CTG ATG CT) was purchased from TIB Molbiol (Berlin). Lipoteichoic acid from *Staphylococcus aureus* was a kind gift from S. Morath (Konstanz, Germany) and was shown to be TLR-2-dependent (37). Highly purified lipopolysaccharide from *Salmonella minnesota* was kindly provided by U. Seydel (Borsten, Germany). Poly(dI-dC) was purchased from Sigma (Schnellendorf, Germany). Pam3CSK was obtained from EMC Microcollections (Tu¨ bingen, Germany). Antibodies specific for phosphorylated MAP kinases, phosphorylated STAT-1, ILBc, and e-Myc were from Cell Signaling Technology (Frankfurt, Germany). Anti-FLAG M2 antibody was obtained from Sigma. Recombinant IFN-γ and GM-CSF were purchased from AbGen (Frankfurt, Germany).

*Mice—*MyD88-deficient mice were obtained from A. Gessner, Erlangen, Germany. IFNAR-I-deficient mice were delivered from B&K Uni-
RESULTS

**TLR-mediated SOCS Induction Differs with Regard to MyD88 Dependence**—TLR activation induces various SOCS proteins; however, the precise mechanisms are still a matter of controversy. We first used MyD88-deficient mice to further gain insight into TLR-mediated SOCS induction (Fig. 1a). MyD88 has been shown to be used as an adaptor by most of the TLRs. Stimulation of peritoneal macrophages with prototypic ligands for different TLRs revealed that induction of SOCS-3 and CIS was completely dependent upon MyD88 in response to all TLR ligands tested. Moreover, peritoneal macrophages showed similar induction of SOCS-3 in response to the different ligands, whereas CIS induction was mainly achieved by LPS and LTA. Interestingly, SOCS-1 induction completely depended on MyD88 in the case of CpG-DNA and LTA stimulation, but induction was mostly preserved in MyD88-deficient mice stimulated with LPS or poly(dI-dC). Thus, a given TLR-dependent gene can be induced by disparate pathways which use distinct adaptor proteins.

**TLR Triggering Induces SOCS in a Direct and Type I IFN-independent Manner**—Next we analyzed a possible role of paracrine acting type I IFN in TLR-mediated induction of SOCS, in particular, SOCS-1. Therefore, we used a transwell system with LPS-responsive C3H/HeN macrophages and LPS non-responsive C3H/HeJ cells in the respective chambers (Fig. 1b). Upon stimulation with LPS, induction of mRNA for the direct target gene TNF-α was only observed in the C3H/HeN macrophages. In contrast, IP-10, which is induced indirectly via secreted type I IFN, showed increased expression in both macrophage populations. SOCS-1 behaved like TNF-α, thus, ruling out a significant contribution of paracrine mediators. CpG-DNA as control showed induction of the different genes in all cells. In addition, we stimulated type I IFN receptor-deficient mice (Fig. 1c). All TLR stimuli induced SOCS-1, SOCS-3, and CIS in dendritic cells. SOCS-3 and CIS induction showed no differences in IFNAR-I/−/− mice as was the case for TNF-α. SOCS-1 induction was not affected in the case of the control stimulus IFN-γ, yet showed a slight, but not significant, decrease in response to LPS and CpG-DNA. In addition, no major role for type I IFN in TLR-mediated SOCS induction could be identified in dendritic cells.

**TLR-1, -2, -3, -4, and -9 Ligands Induce Secondary Type I IFN-dependent Signaling**—To further study the significance of SOCS for TLR signaling, we first assessed whether the concept of direct and indirect type I IFN-dependent TLR signaling (see Fig. 7) holds true for different TLR ligands, a matter that is controversial in the literature. First, we examined induction of transcripts for IFN-β as well as IFN-β-dependent IP-10 in comparison with the direct target gene TNF-α (Fig. 2a). In general, all of the tested TLR ligands (which have been shown to be devoid of contaminants earlier) were able to induce IFN-β and IP-10. The concentrations of the respective stimuli were chosen to give nearly equal TNF-α induction. However, poly(dI-dC) was more efficient in IFN-β induction, and LPS, as well as poly(dI-dC), also induced more IP-10 transcription (in the range of 15-fold more than with CpG-DNA). The results were confirmed by Western blot with phosphotyrosine-specific STAT-1 detection (Fig. 2b). Upon prolonged TLR stimulation, STAT-1 activation could be observed with LPS, CpG-DNA, or poly(dI-dC). Upon prolonged TLR stimulation, STAT-1 activation could be observed with LPS, CpG-DNA, or poly(dI-dC). Finally, cells were stained with PE-labeled secondary antibody (Dako, Hamburg, Germany). Cells were analyzed on a Partec PAS flow cytometer (Dako).

**FIG. 1.** TLR ligands induce SOCS in a direct manner with differing needs of MyD88. a, peritoneal macrophages from wild type or MyD88−/− mice were stimulated with 10 μg/ml LTA, 5 μg/ml poly(dI-dC), 100 ng/ml LPS, or 300 nM CpG-DNA for 5 h. Expression levels for SOCS-1, SOCS-3, and CIS mRNA were determined by quantitative RT-PCR (n = 2, mean ± S.D.). b, peritoneal macrophages from C3H/HeJ and C3H/HeN mice were stimulated in a transwell system with either LPS or CpG as above. Expression levels of SOCS-1, TNF-α, and IP-10 were determined by quantitative RT-PCR (n = 3, mean ± S.D.). c, dendritic cells from IFNAR-1/−/− or wild type mice were stimulated with either LTA, LPS, CpG-DNA, or 30 units/ml IFN-γ and examined as above (n = 2, mean ± S.D.).

LTA in C57Bl/6 macrophages. Again, LPS was more efficient than LTA or CpG-DNA. Results were corroborated with C3H/HeN mice; however, in this case CpG-DNA was much more active, arguing for strain-specific differences in type I IFN signaling. C3H/HeJ mice confirmed that indeed STAT-1 acti-
vation by lipoteichoic acid was not due to LPS contaminants. Moreover, STAT-1 activation differed with respect to MyD88 dependence, whereas CpG-DNA and LTA were completely dependent on MyD88. Finally, transwell experiments confirmed that STAT-1 activation is due to the paracrine action of a secreted factor, most probably type I IFNs (Fig. 2c).

**FIG. 2.** Activation of indirect type I IFN signaling in response to TLR stimulation. Peritoneal macrophages from C57Bl/6 mice were stimulated with 300 nM CpG-DNA, 100 ng/ml LPS, 5 μg/ml poly(dI-dC), 10 μg/ml LTA, or 10 μg/ml Pam3CSK4 for 6 h. a, expression of mRNA for IFN-β, IP-10, and TNF-α was determined relative to β-actin (n = 3, mean ± S.D.). b, lysates were prepared and equal amounts were blotted with phosphospecific STAT-1 Ab. c, peritoneal macrophages were placed in a transwell system (0.4 μm) and stimulated and analyzed as above.

**SOCS Proteins Indirectly Inhibit TLR Signaling**

**Generation of Macrophages with Stable Overexpression of SOCS**—To evaluate a possible role of SOCS in TLR signal transduction we elected to use an overexpression system. To that end, we generated RAW 264.7 macrophage clones with stable overexpression of the respective SOCS molecules. Overexpression at the mRNA level was carefully monitored in different clones (Fig. 3a), and clones with specific and high overexpression were selected for further experiments. Moreover, protein overexpression was also controlled with either FLAG or c-Myc antibodies (data not shown). Untransfected macrophages showed expression of SOCS-1, SOCS-3, and CIS but not of SOCS-2 in response to LPS (Fig. 3b). However, expression levels of SOCS-1 and SOCS-3 were lower than with the stably transfected cells, while CIS levels were comparable. Thus, the stable transfectants had SOCS expression levels at least equal to or higher than LPS activated cells. SOCS proteins at the selected expression levels were functional, since they inhibited prototypical JAK/STAT-dependent cytokine signaling, as shown for SOCS-1-mediated IFN-γ inhibition (Fig. 3c and data not shown).

**SOCS Inhibit Indirectly, but Not Directly, Induced Genes upon TLR Stimulation**—Using RAW 264.7 macrophages with stable overexpression of the various SOCS proteins, we next analyzed the responsiveness toward TLR stimulation. Neither LPS- nor CpG-DNA-mediated TNF-α secretion were significantly inhibited by expression of the various SOCS proteins (Fig. 4a and c). Although single clones existed with slightly differing TLR sensitivity, no significant overall differences became obvious upon repeated experiments. The same results were also observed for lipoteichoic acid and Pam3CSK4 (data not shown). Although iNOS has been described to be partly dependent on paracrine type I IFN induction, we were not able to observe inhibitory effects of SOCS on TLR-mediated nitrite secretion (Fig. 4b and d). However, when we analyzed the induction of the indirect, IFN-β-dependent gene IP-10, we found that secretion of this chemokine upon LPS or CpG-DNA stimulation was severely inhibited by overexpression of either SOCS-1 or SOCS-3 (Fig. 4c). CIS had also a slightly lower inhibitory potential.

**SOCS-1 Inhibits Induction of IP-10 mRNA upon TLR Stimulation**—To corroborate the results obtained at the protein level, we also examined induction of TNF-α and IP-10 at the mRNA level (Fig. 5). Again, LPS and CpG-DNA were equally active in inducing TNF-α transcription, and this was not influenced by SOCS protein overexpression. However, in accord...
with the above results, SOCS-1 overexpression severely and significantly diminished IP-10 induction in response to TLR-4 and -9 triggering. SOCS-3 and CIS were less effective, yet a slight decrease in induction of IP-10 could also be observed.

**SOCS-1 Inhibits TLR-mediated STAT-1 Activation**—Finally, we analyzed the effects of SOCS overexpression on TLR signaling pathways. In line with the above results, no significant effects of SOCS on LPS or CpG-DNA stimulation were observed for the rapid activation of direct signaling events including phosphorylation of p38, JNK, and ERK as well as degradation of IκBα (Fig. 6a). Again, different cell clones showed equal results in Western blotting. However, when cells were stimulated for prolonged time periods with TLR ligands, STAT-1 activation as measured by tyrosine phosphorylation became observable. SOCS-1 and to a lesser extent SOCS-3 and CIS impaired or abolished TLR-mediated STAT-1 activation (Fig. 6b). The same pattern could be observed for the inhibition of IFN-γ signaling upon direct stimulation. As conflicting reports have been published we confirmed the signaling data with different methods. Neither NFκB reporter gene assays (Fig. 6c) nor NFκB translocation assays (Fig. 6d) were able to show inhibitory effects of SOCS on TLR-induced NFκB activation. In controls, SOCS expression (SOCS-1 and -3) abolished IFN-γ signaling at the chosen concentrations as expected. Using phosphotyrosine-specific antibodies in flow cytometry, we were again able to observe a negative regulatory effect of SOCS on LPS-mediated STAT-1 activation, thus, corroborating the above results (Fig. 6e). Again, SOCS-3 and CIS were only partly inhibitory.

**SOCS-1 Inhibits TLR-mediated CD40 Up-regulation**—Indirect type I IFN signaling has been suggested to participate in regulation of costimulatory molecules on antigen presenting cells. Therefore, we analyzed the effect of SOCS overexpression on CD40 expression. RAW macrophages stably overexpressing SOCS proteins were analyzed for the induction of proinflammatory mediators. Cells were stimulated with indicated amount of either LPS (a, b) or CpG-DNA (c, d) for 24 h. Subsequently TNF-α (a, c) and nitrite (b, d) were measured in the supernatant. Displayed is one out of five typical experiments. e, cells were stimulated with either 100 ng/ml LPS or 100 nm CpG-DNA for 24 h, and IP-10 secretion was determined by ELISA (n = 2, mean ± S.D.).

![Graphs and images showing the effects of SOCS overexpression on TNF-α and nitrite production, IP-10 secretion, and CD40 expression.](http://www.jbc.org/content/early/2017/03/31/jbc.M116.769365/Fig4.pdf)
SOCS Proteins Indirectly Inhibit TLR Signaling

DISCUSSION

Based on the presented experiments, a clear picture of the role of SOCS in TLR signaling can be portrayed (Fig. 7) and delineated as follows. TLR-dependent stimuli are able to induce SOCS proteins in a manner that is independent of paracrine-secreted factors and, especially, independent of type I IFN. Moreover, SOCS induction depends on MyD88 with the exception that TLR-3 and TLR-4 are able to use a second pathway for SOCS-1 induction. In turn, SOCS proteins do not inhibit direct MAP kinase- and NFκB signaling. Thus, TLR signaling negatively influences STAT-1 activation.

Concerning the mode of induction of SOCS after TLR stimulation, we did not find evidence for an exclusive role of secreted type I IFN as reported by another group (13), and thus, we confirm earlier results (10, 11). Moreover, experiments using a transwell approach suggested that secreted factors do not play a dominant role at all. However, while it is conceivable that regulation occurs at the transcriptional level (2, 10, 11, 36), the precise mechanism of SOCS induction still remains elusive, since to date no promoter studies in response to TLR stimulation have been performed. We now extend our previous findings, showing that at least for TLR-2 and TLR-9 induction of SOCS-1, SOCS-3, and CIS is completely dependent on MyD88. However, in the case of LPS and poly(dI-dC), an additional pathway seems to be operative. One of the candidates could be TIRAP, since this mediator is reported to mediate additionally activation by LPS (26, 27). However, no role for TIRAP has been found in TLR-3 signaling (39). Thus, it seems more probable that TRIF/TICAM-1 is involved in SOCS-1 induction by LPS and poly(dI-dC) (30, 31). This signaling pathway is also responsible for IFN-β induction by the latter ligands. Interestingly, we now show that TLR-2 and TLR-9 stimulation also results in IFN-β induction and this is completely MyD88-dependent. Thus, this particular gene (IFN-β) can be activated by different TLR stimuli using different adaptors. This is not only true for the partial overlap of MyD88 and TIRAP but also seems to be the case for TRIF and MyD88. These findings are in line with results from the Akira group showing similar differences in MyD88 dependence for TLR-4 and -9 ligands with regard to IFN-β induction and up-regulation of CD40 (40). Moreover, our results also demonstrate that TLR-2 does not lack the ability for IFN-β induction, since lipoteichoic acid was able to activate this signaling branch. This contrasts with earlier findings (34); however, the lipoteichoic acid used was highly purified and characterized to be strictly TLR-2-dependent (37). In addition, lipoteichoic acid-mediated IFN-β and STAT-1 activation was preserved in C3H/HeJ mice. It has to be stated that, possibly, TLR stimuli show different capacities to activate this signaling pathway yet there is no doubt that TLR-2 signaling itself is able to induce STAT-1 activation. The results are in line with a model proposed by O'Neill and Hamilton (24), which reconciled the role of IFNs in TLR signaling. Also, this confirms earlier results showing that cycloheximide is able to inhibit LPS-mediated iNOS induction while having no effects on IFN-β (29).

Here we used IP-10 as a model gene that is induced by the secondary IFN-β-dependent TLR pathway, a fact that has been studied in IFNAR-1-deficient mice earlier (40). Similarly, CD40 regulation in dendritic cells has been reported to be dependent on IFN-β (28, 40). We here report that SOCS-1 only inhibits indirect IFN-β signaling in response to TLR stimulation but has no effects on NFκB. Since up-regulation of CD40 was also diminished in SOCS-1 overexpressing cells, this indirectly argues for IFN-β dependence thereby corroborating the cited results. In contrast, we did not find significant effects on CD86 regulation while others have reported that macrophages from TRIF- or IFNAR-1-deficient mice do not display CD86 regulation anymore (28). Whether this is due to cell type-specific differences has not yet been examined.

Regarding the role of SOCS proteins in TLR signaling, we were not able to find evidence for an inhibitory role of SOCS for direct target genes, in contrast to other groups (35, 36). We chose to use an in vitro overexpression system. Overexpression levels were carefully controlled, and SOCS proteins were functional as they inhibited the expected cytokines (e.g. IFN-γ). Using this approach, neither TNF-α nor IL-6 (data not shown) nor nitrite were inhibited by the different SOCS proteins. However, LPS induced IP-10 as a maker of secondary IFN-β action was diminished by SOCS-1, SOCS-3, and CIS expression. Analyzing the respective signaling pathways, we did not find conflicting results but confirmed that while STAT-1 tyrosine phosphorylation was affected by SOCS, no effects could be observed for MAP kinase and NFκB activation. This contrasts the reported finding using NFκB reporter gene assays. The results are difficult to bring together. However, there is indirect evidence that IFN-β signaling has amplificatory effects on...
direct genes in a mode of positive feedback regulation (41, 42). Such positive feedback regulation could be of differing intensity among various cell types or depending on culture conditions. Indeed, we have preliminary results showing that some macrophage cell lines exhibit reduced TNF-α/H9251 induction upon addition of neutralizing type I IFN antiserum. Thus, it could be that the experimental conditions between the groups differed with respect to actions of IFN-β/H9252. Indeed, it has been shown that Tyk2 knock-out mice, which have a defect in IFN-β/H9252 signaling, show a decreased LPS sensitivity in a LPS shock model (43). This argues for a much more important role of auto/paracrine type I IFN signaling in vivo. This could also explain the differences between our results using in vitro overexpression and the observed effects with SOCS-1-deficient animals. Furthermore, it has been reported that SOCS-1/STAT-1 double-deficient mice show a marked increase in LPS shock resistance as compared with SOCS-1 knock-out mice (36). Although it was stated that these mice still were more susceptible than wild type mice, the differences were only minor suggesting that SOCS-1 effects, independent of STAT-1 inhibition, also play only a minor role in vivo.

Additional controversy resulted from the fact that we did not find a role of SOCS for TLR induced nitrite induction. Others,

FIG. 6. SOCS effects on TLR signaling pathways. a, RAW macrophages with stable overexpression of SOCS were stimulated with 100 ng/ml LPS or 1 μM CpG-DNA for 30 min. Equal amounts of protein lysates were blotted and probed with phosphospecific abs for ERK, JNK, and p38 MAP kinase as well as IκBα. b, cells were stimulated with 100 ng/ml LPS, 0.3 μM CpG-ODN for 4 h or with 10 units/ml IFN-β for 15 min. Lysates were probed with phosphospecific STAT-1 Ab and subsequently reprobed with STAT-1 Ab recognizing total amount of STAT-1. c, RAW 264.7 macrophages were transfected with either the NFκB-dependent ELAM or the STAT-1-dependent IFP53 reporter together with SOCS expression plasmids. Cells were stimulated with either 100 ng/ml LPS or 30 units/ml IFN-γ for 6 h. Induction of the luciferase reporter gene was measured. Experiments were normalized to mock transfected controls (n = 3, mean ± S.D.). d, macrophages with stable overexpression of SOCS were activated with 100 ng/ml LPS for 45 min. Activation of NFκB was measured by an ELISA-based method in nuclear extracts (shown is one of two experiments). e, SOCS overexpressing cells were stimulated for 4.5 h with 100 ng/ml LPS. Cells were stained with a phosphospecific STAT-1 Ab, and mean fluorescence intensity was measured in a flow cytometer. The given stimulation index is the n-fold induction toward non-stimulated control cells (n = 3, mean ± S.D.; *, p < 0.05).

TABLE I

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<th>CD40</th>
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45 min. Activation of NFκB was measured by an ELISA-based method in nuclear extracts (shown is one of two experiments). SOCS overexpressing cells were stimulated for 4.5 h with 100 ng/ml LPS. Cells were stained with a phosphospecific STAT-1 Ab, and mean fluorescence intensity was measured in a flow cytometer. The given stimulation index is the n-fold induction toward non-stimulated control cells (n = 3, mean ± S.D.; *, p < 0.05).
using substitution or depletion of IFN-β (34, 41), have found that iNOS induction depends at least partly on the IFN-β pathway in TLR signaling. However, as this pathway is markedly diminished in SOCS-1 expressing macrophages, one would assume that iNOS induction upon TLR triggering should be diminished. Nevertheless, we failed to observe effects of SOCS-1 on TLR-induced nitrite at an early time point of 24 h. This is in line with observations that IFN-β null mice have equal levels of nitrite in response to LPS as compared with wild type mice in vivo (43). Based on these findings we suggest that early nitrite generation is induced in a direct manner but can be enhanced at later time points by secondary IFN-β action, similar to other genes as described previously (42).

Finally, if one argues for an existing role of SOCS-1 in LPS signaling, clear mechanisms for NFκB affection should be identified. So far only one group was able to show a weak SH2-depletion of IFN-β signaling, clear mechanisms for NFκB affection should be identified. So far only one group was able to show a weak SH2-depletion of IFN-β signaling. However, as this pathway is marked downregulation, thus, contributing to NFκB inhibition. Nevertheless, we failed to observe effects of this pathway in TLR signaling. However, as this pathway is marked downregulation, thus, contributing to NFκB inhibition. Nevertheless, we failed to observe effects of this pathway in TLR signaling. However, as this pathway is marked downregulation, thus, contributing to NFκB inhibition. Nevertheless, we failed to observe effects of this pathway in TLR signaling. However, as this pathway is marked downregulation, thus, contributing to NFκB inhibition. Nevertheless, we failed to observe effects of this pathway in TLR signaling. However, as this pathway is marked downregulation, thus, contributing to NFκB inhibition. Nevertheless, we failed to observe effects of this pathway in TLR signaling. However, as this pathway is marked downregulation, thus, contributing to NFκB inhibition. Nevertheless, we failed to observe effects of this pathway in TLR signaling. However, as this pathway is marked downregulation, thus, contributing to NFκB inhibition.
Suppressor of Cytokine Signaling (SOCS) Proteins Indirectly Regulate Toll-like Receptor Signaling in Innate Immune Cells
Andrea Baetz, Markus Frey, Klaus Heeg and Alexander H. Dalpke

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