Suppressor of Cytokine Signaling 2 Is a Feedback Inhibitor of TLR-Induced Activation in Human Monocyte-Derived Dendritic Cells

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Dendritic cells (DCs) are key players in initiating and directing the immune response. Therefore, their activation state and functional differentiation need to be tightly controlled. The activating stimuli and their signaling networks have long been an area of focus in DC research. Recent investigations have also shed light on the mechanisms of counterregulation and fine-tuning of DC functions. One class of proteins involved in these processes is the family of suppressors of cytokine signaling (SOCS), whose members were originally described as feedback inhibitors of cytokine-induced JAK/STAT signaling. Essential roles in DC function have been assigned to SOCS1 and SOCS3. In this article, we show that SOCS2 also is involved in DC regulation. In human and in murine DCs, SOCS2 is a highly TLR-responsive gene, which is expressed in a time-delayed fashion beginning 8 h after TLR ligation. Functionally, silencing of SOCS2 in DCs results in hyperphosphorylation of STAT3 at later time points. As a consequence, SOCS2-deficient DCs secrete increased amounts of the cytokines IL-1β and IL-10, both being transcriptional targets of STAT3. We propose a model in which SOCS2 acts as a negative regulator of TLR-induced DC activation. The delayed expression of SOCS2 provides a mechanism of late-phase counterregulation and limitation of inflammation-driving DC activity. The Journal of Immunology, 2011, 187: 2875–2884.

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Abbreviations used in this article: CIS, cytokine-inducible SH2-containing protein; DC, dendritic cell; GH, growth hormone; KIR, kinase inhibitory region; moDC, monocyte-derived DC; poly I:C, polyriboinosinic:polyribocytidylic acid; qRT-PCR, quantitative RT-PCR; RPLP0, large ribosomal protein P0; SH2, Src homology 2; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling.

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overshooting of immune activation (14). SOCS1 deficiency in APCs results in hyperactivation and consecutive hyper-Th1 responses (15).

In contrast, SOCS3 deficiency furnishes DCs with a tolerogenic phenotype. In response to many gp130 cytokines, SOCS3-dependent signal termination limits the duration of STAT3 activation. Opposed to that, IL-10 signaling is not sensitive to SOCS3 inhibition and induces long-lasting phosphorylation of STAT3. In the absence of SOCS3, a wide array of gp130 cytokines is able to induce prolonged STAT3 activation and to mimic IL-10 stimulation. As a consequence, these cytokines share the anti-inflammatory properties of IL-10 (16–18).

SOCS2 was originally described as a feedback inhibitor of the growth hormone (GH)/insulin-like growth factor axis, which is reflected by the high growth phenotype of SOCS2-deficient mice (19). Surprisingly, SOCS2 transgenic mice also show an increase in body weight, suggesting a dual role for SOCS2 in the GH signaling cascade. This is supported by in vitro experiments, in which only low-to-intermediate levels of SOCS2 show inhibitory action on GH signaling, whereas high levels of ectopic SOCS2 expression even increase STAT5 activation in response to GH stimulation (20, 21). SOCS2 expression is induced by a number of different cytokines and hormones in many cell types (22). In addition, dioxin and the lipid mediator lipoxin A4 have been described to stimulate SOCS2 expression in B cells and DCs, respectively, each of them dependent on aryl-hydrocarbon receptor activation (23, 24). Based on forced expression, a regulatory potential for SOCS2 has been suggested in several pathways; however, the physiological relevance of these results is controversial. Because SOCS2 lacks a KIR domain, the inhibitory function of SOCS2 is dependent on competitive binding via its SH2 domain and, even more important, on the proteosomal degradation of the proteins with which it interacts (8). Apart from altered GH signaling, SOCS2-deficient mice have also been shown to exhibit alterations in their immune system. They display overshooting immune reactions in a model of toxoplasmosis because of a failure to counterregulate DC activation. SOCS2-dependent TRAF6 degradation in APCs was suggested as the underlying mechanism (25). However, that study was recently retracted and the proposed mechanism remains in doubt (26). Nevertheless, the main conclusion that SOCS2 plays a regulatory role in DC activation appears to be well supported.

In this study, we show that SOCS2 is induced as a consequence of TLR stimulation in both human and murine DCs. Compared with SOCS1 and SOCS3, which are upregulated within 1 h after LPS treatment, SOCS2 shows a time-delayed expression starting ~8 h poststimulation. Despite this delay, SOCS2 is a direct target of TLR signaling, for its expression is robust under conditions of protein-synthesis inhibition. Silencing of SOCS2 in DCs leads to increased cytokine secretion, predominantly of cytokines with a late and more sustained expression pattern, like IL-10 and IL-1β. Cytokines with early and transient expression, such as TNF-α or IL-6, were not affected. Moreover, elevated, IL-10–independent, STAT3 phosphorylation was observed in SOCS2–deficient DCs. In conclusion, our data suggest an inhibitory function for SOCS2 in TLR ligand-induced DC activation.

Materials and Methods

All studies involving human material were conducted in accordance with the guidelines of the World Medical Association’s Declaration of Helsinki.

Generation of human monocyte-derived DCs

Monocyte-derived DCs (moDCs) were generated according to the slightly modified standard protocol (27). In brief, PBMCs were isolated from buffy coats from healthy donors (kindly provided by Transfusionsmedizin der Paracelsus Medical University, Salzburg, Austria) over Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) by density gradient centrifugation, according to the manufacturer’s instructions. PBMCs were washed twice in RPMI 1640, and cells were allowed to adhere for 90 min. Adherent monocytes were washed extensively with warm RPMI 1640 to remove all nonadherent cells and cultured for 7 d in DC medium (RPMI 1640; PAA, Pasching, Austria), 10% FCS (PAA), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME (all from Life Technologies Laboratories, Grand Island, NY), and stimulated with 50 ng/ml GM-CSF and 50 ng/ml IL-4 (generous gift from Nowartis, Vienna, Austria). At day 3, cells were fed with 1 vol DC medium containing fresh cytokines. After 6 d, cells were harvested and replated in DC medium without cytokines. At this stage, cells were phenotyped by flow cytometry and were routinely ≥90% CD14+ CD1α+.

Isolation of primary human blood DCs and CD4+CD45RA– naive T cells

CD14– DCs were isolated from CD19-depleted PBMCs using a BDCA1+ Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. CD4+CD45RA– T cells were isolated using the untouched naive CD T cell isolation kit II (Miltenyi Biotec), according to the manufacturer’s instructions. Isolated cells were phenotyped by FACS.

Generation of thioglycolate-induced mouse peritoneal macrophages

cDNAs from both untreated and LPS-stimulated, thioglycolate-induced peritoneal macrophages were kindly provided by G. Schwamberger. Macrophages were obtained according to standard protocols. In brief, mice were ip injected with 1 ml 1% thioglycolate solution. After 4 d, mice were killed and peritoneal lavage cells were collected. Cells were plated in IMDM (PAA) with antibiotics for 3 h and washed extensively afterward. The adherent fraction was used for further stimulation as indicated.

Generation of mouse bone marrow-derived DCs

Bone marrow-derived DCs were generated according to standard protocols. In brief, bone marrow cells were isolated from femurs of BALB/c mice (Charles River). Cells were differentiated in DC medium supplemented with 5% mouse GM-CSF–conditioned supernant (mouse DC medium) for 9 d. At days 3 and 6, half of the medium was replaced with fresh mouse DC medium. After 9 d, cells were sedimented and replated for further stimulation in mouse DC medium. Cells were routinely phenotyped for CD11c expression by means of FACS analysis.

TLR ligands and cell culture reagents

Cells were stimulated with Escherichia coli LPS 055:B5 (Sigma-Aldrich), Pam3CSK4, FSL-1, Flagellin (all from InvivoGen, San Diego, CA), R848 (Alexis Biochemicals, Lausen, Switzerland), immunostimulatory CpG oligodeoxynucleotide C274 (as described in Ref. 28, phosphorothioate modified), and polyribonosinic:polyribocytidylic acid (poly I:C) (Sigma-Aldrich). For the control of STAT3 phosphorylation in response to IL-10 stimulation, cells were incubated with 25 ng/ml recombinant human IL-10 (Immunotools, Friesoythe, Germany). Where indicated, experiments were conducted in the presence of the IL-10R–blocking Ab anti–CD127 (BD Pharmingen) at 20 μg/ml for inhibition of autocrine/paracrine IL-10 stimulation. The STAT3 inhibitor Static (6-nitrobenzoylβ-thiophene-1, 1-dioxide) was purchased from Calbiochem (Darmstadt, Germany).

ELISA

For each condition, 5 × 10^5 cells were plated per six wells in 1.5 ml DC medium. Supernatants of DCs were collected and stored at −80°C until analysis. Cytokine secretion was measured by using commercially available ELISA kits for TNF-α, IL-6 (both from PepeTech, Eubio, Vienna, Austria), IL-12p70 (BD Pharmingen, Erembodegen, Belgium), IL-1β, and IL-10 (both from R&D Systems, Biomedica, Vienna, Austria). ELISA results are shown as normalized secretion. Because of heterogeneous absolute values in different donors, LPS-stimulated secretion in control cells was set as 100%.

Western blot analysis

Cells were sedimented, washed, and lysed either directly in 2× SDS sample buffer (Bio-Rad, Vienna, Austria) or in ice-cold Nonidet P-40 lysis buffer (Invitrogen, Lofer, Austria) supplemented with 1 mM PMSF and Complete Mini proteinase inhibitor mixture (Roche) for 30 min. Nonidet P-40 lysates were cleared of debris by centrifugation. Equal amounts of protein were...
separated by 4–12% SDS-PAGE (Invitrogen) under reducing conditions, blotted on nitrocellulose membrane (Bio-Rad), and blocked with 5% nonfat dry milk (Carl-Roth, Karlsruhe, Germany) in TBS with 0.05% Tween 20. All Abs were purchased from Cell Signaling Technology (Danvers, MA) and used according to the manufacturer’s instructions. Western blot quantification was done with ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Quantitative real-time PCR

Quantitative RT-PCR (qRT-PCR) was done as described previously (29). In brief, total RNA was isolated with TRizol (Invitrogen, Lofer, Austria) or a Nucleospin RNA II kit (Macherey-Nagel, Dürren, Germany) according to the manufacturer’s instructions. For qRT-PCR, 2 μg total RNA was reverse-transcribed using RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Roth, Germany), following the manufacturer’s protocol. qRT-PCR was performed in a Rotorgene 3000 (Corbett Research) with ready-to-use 2x iQ SYBR Green Supermix (Bio-Rad). Primers were designed by using Vector NTI software (Invitrogen) to amplify targets ranging in size from 120 to 200 nucleotides with annealing temperatures of 65°C. Target specificity was assessed by product sequencing and routine recording of melting curves. Sequences of the primers are listed in Table I. mRNA content (m) was calculated using the formula $m = 2^{-\Delta C_t}$, where $\Delta C_t$ represents the difference between the gene of interest and the reference gene, large ribosomal protein P0 (RPLP0). The fold-change value represents the quotient of the mRNA content of the induced sample and that of the corresponding noninduced sample. Analogous to the ELISA results, glass fiber filters (MACH III M cell harvester; TOMTEC), sealed with scintillation sheets (MeltiLex), and analyzed in a Microbeta 1450 scintillation counter (PerkinElmer). Small interfering RNA and MLR

For knockdown experiments, we used the validated SOCS2 stealth RNAi Duopack (Invitrogen) and a scrambled sequence oligo with similar GC content as a control oligo. Cells were transfected with Lipofectamine RNAiMax reagent (Invitrogen) according to the manufacturer’s guide- lines. In brief, at day 6 of differentiation, 5 × 10^5 cells were plated in antibiotic-free DC medium and transfection with 100 pmol/well small interfering RNA (siRNA). After overnight incubation, the medium was supplemented with antibiotics and the cells were used for further experiments. Transfection efficiency was routinely >90%, as assessed by flow cytometry with fluorescent control siRNA oligonucleotides (BlockIT; Invitrogen). Knockdown efficacy was analyzed by means of qRT-PCR and Western blotting. For allogeneic MLRs, 1 × 10^5 naïve CD4+CD45RA+ T cells were cultured with DCs at a ratio of 30:1 (T cells/DCs) in 96-well, round-bottom plates. After 4 d of coculture, cells were pulsed with 1 μCi [3H]thymidine for 16 h. Cells were transferred onto glass fiber filters (MACH III M cell harvester; TOMTEC), sealed with scintillation sheets (MeltiLex), and analyzed in a Microbeta 1450 scintillation counter (PerkinElmer). Analogue to the ELISA results, fold-change mRNA values were calculated for the upregulated SOCS1-3 and human CIS (CISH). Data show representative results from one of five donors.

### Table I. qRT-PCR primer sequences

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<th>Target Gene</th>
<th>Strand</th>
<th>Sequence</th>
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<td>5’-TCAGTGGAGAGTTGGCCAGTCTTCAGTC-3’</td>
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<td>Socs2</td>
<td>Forward</td>
<td>5’-GGCACCATTGAAATCCTGAGTGATGTG-3’</td>
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**FIGURE 1.** Expression profile of SOCS family members in human moDCs. moDCs were harvested and replated at day 7 of differentiation in DC medium at 2.5 × 10^5 cells/ml. Cells were either left untreated or stimulated with 100 ng/ml E. coli LPS for 24 h. mRNA expression levels of all SOCS family members are shown as relative expression to RPLP0 (A) by means of qRT-PCR. Fold-change mRNA values were calculated for the upregulated SOCS1-3 and human CIS (CISH) (B). Data show representative results from one of five donors.
proliferation results are shown as normalized proliferation. Proliferation stimulated by LPS-treated control cells was set as 100%.

Flow cytometry
All FACS analyses were done on a FACS Canto II instrument (Becton Dickinson) using FACS Diva software for acquisition and analysis. FACS Abs to human Ags (CD1a, CD14, CD40, CD80, CD86, HLA DR, PD-L1, PD-L2, CCR4, CCR7) were all purchased from BD Pharmingen. Staining was done according to standard procedures. Cells were then washed in PBS/FCS and fixed in 0.1% paraformaldehyde until analysis.

Results
SOCS2 is expressed in APCs in response to TLR ligands
SOCS1, SOCS3, and CIS were described to be TLR-responsive genes, and specific functions for these proteins in DCs have been established (10, 11, 13–16, 30). Therefore, we screened human moDCs for expression of all SOCS family members (for primer sequences, see Table I). In a long-term qRT-PCR kinetics experiment, we observed that, in addition to SOCS1, SOCS3, and CIS, SOCS2 is a highly LPS-responsive gene. For SOCS4-7, no significant expression was detectable in LPS-treated DCs (Fig. 1).

In contrast with SOCS1 and SOCS3, SOCS2 was upregulated in a delayed fashion, beginning ∼8 h after LPS stimulation and peaking at 24 h after LPS treatment (Fig. 2A). To verify these results, we also analyzed the expression of SOCS2 at the protein level, and the results fully agree with the data from the initial qRT-PCR screen: SOCS2 protein remained undetectable up to 8 h; thereafter, increasing protein expression was detectable, and SOCS2 levels continued to accumulate up to 48 h (Fig. 2B). To exclude potential contributions of residual impurities in our DC cultures, we also analyzed expression in CD1a+ FACS-sorted DCs (>99%) and obtained the same results (Supplemental Fig. 1). Because moDCs represent inflammation-induced DCs and do not share all features with conventional DCs (31), we chose to isolate primary CD1c+ blood DCs to confirm the results obtained with the moDCs (Fig. 3A, 3B).

For further validation of our data, we also measured SOCS2 expression in murine APCs. LPS stimulation of mouse bone marrow-derived DCs and thioglycolate-induced mouse peritoneal macrophages resulted in a significant upregulation of SOCS2 mRNA levels. The time course follows a similar delayed expression pattern (Fig. 3C, 3D). The delayed timing of SOCS2 expression suggested the possibility that the MyD88-independent TLR4 pathway, which induces delayed activation of NF-κB and IFN regulatory factor 3 (32), could be responsible for SOCS2 expression. To test this hypothesis, we investigated whether SOCS2 expression is triggered solely by the TLR4 pathway or is equally activated by other TLRs that exclusively use the MyD88-dependent pathway. In addition to the TLR4 ligand LPS, we tested a whole panel of commercially available TLR ligands. moDCs were stimulated with Pam3CSK4 (for TLR1/2), FSL-1 (TLR2/6), poly I:C (TLR3), flagellin (TLR 5), R848 (TLR7, TLR8), and type C CpG-containing oligonucleotides (28) (TLR9). SOCS2 expression was observed in response to stimulation of TLR1/2, TLR2/6, TLR4, and TLR7/TLR8, albeit to varying

FIGURE 2. Time-course experiments show delayed expression of SOCS2. moDCs were harvested and replated at day 7 of differentiation in DC medium at 2.5 × 10⁵ cells/ml. Cells were stimulated with 100 ng/ml E. coli LPS for the indicated times. mRNA expression levels of SOCS1-3 are shown as relative expression compared with RPLP0 by means of qRT-PCR and are representative of all donors analyzed throughout the study (A). Error bars indicate the SD of two independent experiments for the same donor. B, DCs were stimulated with E. coli LPS as indicated, and total cell lysates were subjected to Western blot analyses for SOCS2 and for STAT1 as a control for equal loading. All results are representative of five independent experiments.
extents (Fig. 4). These observations are in line with previous reports that human moDCs do not express TLR5, TLR7, and TLR9, but do express TLR1-4, TLR6, and TLR8 (33, 34). Interestingly, TLR3 stimulation resulted in increased mRNA levels but failed to produce significant levels of SOCS2 protein. Taken together, these results suggest that SOCS2 is a feedback molecule that is induced by all TLRs that signal through the MyD88-dependent pathway, and that its expression is not reliant on the alternative signaling pathway of the LPS receptor TLR4. Notably, although SOCS2 is manifestly induced in all primary human and mouse APCs in response to TLR stimulation, we were not able to observe induction of SOCS2 mRNA in response to LPS in any of the tested monocytic-like, macrophage-like, or DC-like cell lines (THP-1, Monomac-1; RAW, Jaws II). Therefore, primary human moDCs were used for all subsequent experiments.

**SOCS2 is a direct downstream target of TLR ligation**

Although delayed expression of SOCS2 has been repeatedly reported in other studies (35–37), the deferred induction raised the question whether SOCS2 is a direct downstream target of TLR signaling or is stimulated via secreted factors in an autocrine/paracrine fashion. To exclude potential contributions of autocrine cytokine stimulation, we treated immature DCs with different candidate cytokines that are produced by activated DCs themselves (IL-1, IL-6, IL-12, IL-10, TNF-α); however, none of the tested cytokines nor the combination of them was able to induce significant levels of SOCS2 mRNA in the absence of LPS (data not shown). To further address this question, we stimulated the cells with LPS in the presence of the protein synthesis inhibitor cycloheximide, and even though the time kinetics was slightly changed, SOCS2 expression was robust in these settings (Supplemental Fig. 2). Because DCs are rather sensitive to cycloheximide treatment, we had to use a relatively low concentration of the drug (20 μM); nevertheless, protein synthesis inhibition was still efficient, as verified by inhibition of TNF-α production. Nevertheless, a potential contribution of preformed intracellular or membrane attached cytokines (38) cannot absolutely be excluded.

**Silencing of SOCS2 in DCs leads to increased secretion of IL-10 and IL-1β**

To investigate the functional role of SOCS2 in DCs, we conducted RNA interference experiments. To avoid unintended adverse effects, for example, cellular stress responses like the PKR/IFN response, we used chemically modified oligonucleotides applying the Stealth RNAi technology. Silencing was performed with two different oligos targeting SOCS2 mRNA to minimize the risk for both sequence-dependent and off-target effects. Neither the SOCS2 oligos nor the control oligo caused any DC maturation themselves, as determined by FACS analyses (CD83; CD86) and cytokine ELISA (TNF-α), and showed no transcription of the type I IFN IFN-α2 compared with untransfected cells (data not shown). Silencing efficacy of the siRNA oligos on mRNA levels was ~70%, as determined by qRT-PCR (data not shown); however, SOCS2 protein was hardly detectable, as shown in Western blot analysis of the corresponding cell lysates (Fig. 5B). Analysis of the surface expression of the DC activation-associated markers CD40, CD80, CD86, CD83, CCR4, and CCR7 failed to identify significant differences between SOCS2-silenced DCs and control cells 24 h after LPS stimulation (data not shown). At the same time point, we analyzed supernatants by ELISA and observed increased levels of IL-1β (100% = 115 pg/ml) and IL-10 (100% = 26 pg/ml) in...
SOCS2-deficient DCs, whereas TNF-α (100% = 1.3 ng/ml) and IL-6 (100% = 1.4 ng/ml) levels remained unaffected (Fig. 5A). For a better understanding of the observed changes in cytokine production, we conducted time-course experiments and measured the actual mRNA levels by qRT-PCR, as well as the cumulative cytokine secretion quantified by ELISA for each time point. As reported previously, TNF-α and IL-6 showed an early-to-intermediate transcription profile (39) (data not shown), which was completely extinguished before substantial amounts of SOCS2 were produced in control cells. In contrast, IL-1β and IL-10 showed delayed and sustained transcription. IL-1β and IL-10 levels were increased after SOCS2 silencing, both at the transcriptional level and in terms of cumulative protein secretion (Fig. 6). The observations corroborate our hypothesis that SOCS2 specifically regulates time-delayed processes during DC maturation, whereas the early response, including IL-6 and TNF-α secretion, is not affected. In addition, a slight reduction of IL-12p70 protein level was observed under SOCS2 knockdown, most likely because of heightened IL-10 secretion into the culture medium (40).

**FIGURE 5.** Silencing of SOCS2 results in increased secretion of IL-1β and IL-10. moDCs were transfected with control siRNA or with SOCS2 siRNA duplex S2 #1 and S2 #2, and either left untreated (black bars) or stimulated with E. coli LPS for 24 h (gray bars). Cytokine secretion was determined by means of ELISA (A). Normalized secretion was calculated with secretion of LPS-treated control cells (CTRL-LPS) equals 100%. Results represent mean and SD of four independent donors (*p < 0.05, **p < 0.005). At the same time points, total cell lysates were prepared and analyzed for SOCS2 and STAT1 as controls for equal loading by means of Western blot (B).

SOCS2-deficient DCs show hyperactivation of late-phase STAT3

The observed increased cytokine secretion with specificity for IL-10 and IL-1β could indicate that SOCS2 targets a transcription factor involved in regulating the expression of both cytokines. A promising candidate was STAT3, because it was previously described to play vital roles in the regulation of IL-6, IL-10, and IL-1β in response to LPS stimulation (41, 42). To test whether STAT3 activation is necessary for the expression of these cytokines in human moDCs, we treated the cells with the STAT3-specific inhibitor compound Stattic and found concentration-dependent repression of IL-1β, IL-6, and IL-10 transcription, whereas TNF-α was not sensitive toward STAT3 inhibition (Supplemental Fig. 3).

To analyze potential alterations of transcription factor activation, we repeated the time-series experiments and subjected cell lysates to Western blotting for analyses of phospho-STAT3, phospho-STAT1, phospho-ERK1/2, phospho-p38, and phospho-IκB. As hypothesized, significantly enhanced phosphorylation of STAT3 was observed, predominantly at late time points (Fig. 7A, 7B). Although different donors showed slightly variant time kinetics of STAT3 hyperphosphorylation, a robust effect was observed for all donors at 24 h of LPS treatment (Fig. 7C). This also correlates with our cytokine-secretion data, where significant enhanced production of IL-1β and IL-10 is exclusively observed at later time points (Fig. 6B). The absent effect of overloud STAT3 activation in SOCS2-silenced DCs on IL-6 production is explained by the different timing of SOCS2 and IL-6 transcription. As a matter of fact, IL-6 transcription peaks and returns to basal levels before significant expression of SOCS2 is detected (data not shown) (39).
not able to observe any differences in early activation of the NF-κB or MAPK pathway (data not shown). These results are consistent with the time course of SOCS2 expression and the absence of effects on early targets of TLR signaling such as TNF-α.

In interpreting these results, it has to be considered that the interrelationship between IL-10 and STAT3 is ambiguous; IL-10 is not only regulated by STAT3, it also uses STAT3 as a major effector molecule for signal transduction. To test whether increased pSTAT3 levels are the origin of increased IL-10 and IL-1β production and not a consequence of heightened IL-10 secretion, the experiments were repeated in the presence of an IL-10R–blocking Ab. Consistent with our hypothesis, STAT3 hyperphosphorylation in SOCS2-deficient cells persisted despite abrogated IL-10 signaling; although the absolute values show reduced levels in case of anti–IL-10R treatment, the effect of STAT3 hyperphosphorylation cannot be attributed to autocrine IL-10 stimulation (Fig. 7D, 7E).

**SOCS2-deficient DCs induce enhanced proliferation in naive CD4+ T cells**

SOCS2-deficient DCs are characterized by both increased secretion of IL-1β, a proinflammatory cytokine, and an increased secretion of IL-10, an anti-inflammatory cytokine. To analyze the consequences of this dichotomy in the cytokine profile on subsequent T cell activation, we performed allogeneic proliferation assays with naive CD4+CD45RA+ T cells. As expected, there is no influence of the SOCS2 knockdown in the proliferation of T cells cocultured with immature DCs, for there is virtually no expression of SOCS2 in DCs in absence of LPS treatment (Fig. 2). By contrast, LPS-activated SOCS2 knockdown DCs induced a 17% increased T cell proliferation compared with LPS-activated control DCs (100% = 2165 cpm), as determined by [3H]thymidine incorporation (Fig. 8). In these allogeneic in vitro settings, we were not able to observe any differences in the production of T cell-derived cytokines. Cultures with both control DCs and SOCS2-deficient DCs resulted in an IFN-γ–dominated Th1 response (data not shown).

**Discussion**

The mechanisms underlying the activation of DCs have been a subject of intense research for decades and are now reasonably well understood. More recently, greater attention has been paid to the counterregulation of these activation processes. Balancing of the level of immune activation, which is necessary for the clearance of pathogens and the control of inflammation, is crucial to prevent excessive damage to the host. Therefore, understanding the mechanisms that limit inflammation is of utmost importance for therapeutic approaches that target immune pathologies caused by dysregulated immune responses.

TLR signaling is one of the major pathways for inducing innate immune activation. Mechanisms of counterregulation target each...
level of the signaling cascade and include soluble decoy receptor variants (43), alternatively spliced adaptor molecules (MyD88s) (44, 45), and a kinase-inactive form of the IL-1R–associated kinase family (IRAK-M) (46). More recently, SOCS1 was shown to be crucially involved in the regulation of TLR4 signaling, because it uses proteasomal depletion of the adaptor molecule MAL as a regulatory mechanism (13). Furthermore, SOCS3 is able to tag the TRAF6/TAK1 complex for proteasomal degradation, thereby limiting signal duration (47). Whereas SOCS1 and SOCS3 have been extensively studied in immune cells, most studies of SOCS2 addressed its function in GH/insulin-like growth factor-mediated STAT5 activation (22). It has been shown that SOCS2 binds to phospho-tyrosine residues of the GH receptor, and that STAT5 inhibition is dependent on the SOCS-box (48). Crystal structures show interaction of the SOCS-box with elongin B and C, thus forming a ternary ubiquitin ligase complex able to mark interacting proteins for degradation (49).

In this study, we showed that SOCS2 expression in DCs is induced in direct response to TLR activation. Although time-delayed expression appears to be a characteristic of SOCS2 (35–37), we determined that SOCS2 expression is independent of indirect autocrine stimulation, for it requires no de novo protein synthesis. Moreover, SOCS2 expression could be observed in response to multiple TLR ligands, indicating that only MyD88-dependent signaling is necessary for its transcription. Despite limited data pertaining to the function of SOCS2 in the immune system, SOCS2 was recently described as the main mediator of the anti-inflammatory capacities of both natural and aspirin-triggered lipoxins. Furthermore, SOCS2-deficient mice suffer from exuberant immune activation in a toxoplasmosis model (24, 25). Our data support the immune-dampening function of SOCS2 in human DCs rather than a coactivating function in TLR signaling, as suggested in a recent publication (50). In our experimental setup, the early transcriptional targets of TLR activation such as TNF-α or IL-6 remained unaffected; however, SOCS2-silenced DCs displayed increased secretion of IL-10 and IL-1β, two cytokines with a late and sustained expression pattern and both transcriptional targets of STAT3 (41, 42). In allogeneic proliferation assays, we could show that the proinflammatory activity of IL-1β dominates over the IL-10 effect, for SOCS2-deficient DCs induce hyperproliferation in allogeneic naive T cells (Fig. 8). Moreover, we were able to demonstrate that, in SOCS2-deficient DCs, the late activation of STAT3 is enhanced and is independent of autocrine IL-10 signaling, which suggests...
that SOCS2 targets an upstream kinase or signaling intermediate responsible for STAT3 activation. It is unlikely that SOCS2-mediated degradation of SOCS3 protein (37) contributes to this effect, for it would lead to decreased levels of STAT3 activation. The proposed regulatory function of SOCS2 in STAT3 activation is in line with a recent study demonstrating that augmented STAT3 activation correlates with hypermethylated–in–gene silencing of SOCS1 and SOCS2 in ovarian and breast carcinomas (51). However, in opposition to a recent report showing hyperactivation of SOCS1 and SOCS2 in ovarian and breast carcinomas (51), we were not able to detect any significant changes in activation of the NF-kB and ERK1/2 pathways (data not shown). Our observations are corroborated by the delayed expression profile of SOCS2, in which neither elevated SOCS2 mRNA nor protein was detectable earlier than 8 h after LPS treatment. The discrepancies might, at least in part, be explained by the use of different methods for siRNA transfection and the types of siRNA used. Furthermore, we suspect that the reduction in all parameters as observed by the previous authors might be indicative of incomplete target specificity in their silencing experiments. These results could also be attributed to siRNA- or electroporation-induced preactivation of the DCs and, therefore, an altered maturation response toward LPS treatment.

In conclusion, our data provide evidence that SOCS2 is a feedback inhibitor of TLR-activated DC maturation. The delay expression allows for a period of unrestricted immune activation followed by a phase of counterregulation and limitation of inflammation-driving activity. However, the molecular target that interconnects SOCS2 expression with limited STAT3 activation, and consequently limited cytokine secretion, remains elusive and needs to be addressed in further studies.

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Disclosures

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References


