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Involvement of Suppressor of Cytokine Signaling-3 as a Mediator of the Inhibitory Effects of IL-10 on Lipopolysaccharide-Induced Macrophage Activation[1]

Chiara Berlato,* Marco A. Cassatella,* Ichiko Kinjyo,[†] Luana Gatto,* Akihiko Yoshimura,[†] and Flavia Bazzoni[2]*

Previous studies have shown that IL-10 can induce the expression of the suppressor of cytokine signaling 3 (SOCS-3) mRNA in human monocytes and neutrophils, suggesting that the capacity of IL-10 to inhibit the expression of LPS-inducible proinflammatory genes may depend on SOCS-3 induction. However, no direct experimental evidence has been provided to support such hypothesis. Herein, we show that stable transfection of SOCS-3 into the mouse macrophage cell line J774 resulted in an inhibition of NO, TNF-α, IL-6, and GM-CSF secretion in response to LPS at levels similar to those exerted by IL-10 in LPS-stimulated wild-type J774. Constitutive SOCS-3 expression also down-regulated the mRNA expression of inducible NO synthase and IL-6 and impaired the production of TNF-α, mainly at a post-transcriptional level. In addition, SOCS-3-transfected cells displayed a constitutive expression of the IL-1R antagonist gene, consistent with the observation that IL-10 enhances IL-1R antagonist mRNA in LPS-stimulated wild-type cells. Furthermore, in peritoneal macrophages harvested from mice carrying heterozygous disruption of the SOCS-3 gene, IL-10 was less effective in repressing LPS-stimulated TNF-α and NO production. Taken together, our data show that SOCS-3 inhibits LPS-induced macrophage activation, strongly supporting the idea that it plays a role in the molecular mechanism by which IL-10 down-modulates the effector functions of LPS-activated macrophages. Finally, we show that forced expression of SOCS-3 significantly suppresses the ability of IL-10 to trigger tyrosine phosphorylation of STAT3. Therefore, SOCS-3 functions both as an LPS signal inhibitor and as a negative feedback regulator of IL-10/STAT3 signaling. *The Journal of Immunology,* 2002, 168: 6404–6411.

T he inflammatory response is a vital component of the host defense; nonetheless, it can be very harmful and potentially fatal if it is not tightly regulated in vivo. Among the various mechanisms conducted to control the inflammatory processes, the activities of IL-10 play a fundamental role. Indeed, the observations that IL-10 prevents the development of certain inflammatory and immune responses in vivo (1) and protects against endotoxin-induced lethality (2–4) as well as the analysis of the IL-10 knockout mice phenotype, which causes enterocolitis and dysregulated inflammatory responses (5–7), fully support the idea that IL-10 protects the host from overwhelming immune/inflammatory responses. Although the immunoregulatory mechanisms exerted by IL-10 appear complex and not yet defined, its anti-inflammatory activity is achieved mainly by inhibiting several functional responses of monocytes/macrophages and neutrophils, including constitutive and IFN-γ-induced MHC class II expression, reactive oxygen intermediates and NO production, phagocytosis, and synthesis of proinflammatory cytokines in response to LPS or other agonists (8, 9).

The intracellular mechanisms by which IL-10 inhibits proinflammatory cytokine production by LPS-activated macrophages remain largely unclear, even though several studies have shown that they are dependent on de novo protein synthesis (10, 11). In this context it has been recently reported that one of the IL-10 target genes is suppressor of cytokine signaling 3 (SOCS-3)[3] (12, 13). The latter protein is a member of a growing family of suppressor of cytokine signaling molecules that were initially described as feedback inhibitors of cytokine signaling pathways and have subsequently gained a widespread role in the negative crosstalk between cytokines and other proinflammatory stimuli (14). A number of studies have demonstrated that SOCS-3 mRNA can be induced by different classes of agonists, including cytokines, hormones, and infectious agents in different tissues (15). The observations that the inhibitory actions of IL-10 and IL-10-mediated induction of SOCS-3 mRNA are temporally associated (12, 13) indicate that SOCS-3 may be a cytoplasmic effector molecule of IL-10-mediated inhibitory mechanisms (16).

To determine whether the mechanisms by which IL-10 negatively regulates the proinflammatory responses of macrophages to LPS occur via SOCS-3 protein expression, we generated a macrophage cell line stably expressing SOCS-3 cDNA. In this work we show that in SOCS-3-transfected macrophages the production of LPS-induced NO, TNF-α, IL-6, and GM-CSF is inhibited to an extent similar to that observed in wild-type cells treated with IL-10

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[3] Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; ERK1/2, extracellular signal-regulated kinase 1/2; IL-1ra, IL-1R antagonist; iNOS, inducible NO synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; RPA, ribonuclease protection assay; SAPK, stress-activated protein kinase.

and LPS. In contrast, expression of the IL-1R antagonist (IL-1ra) gene in SOCS-3-transfected macrophages is constitutive and remained inducible by LPS. We also show that the inhibitory activities of IL-10 on LPS-induced NO and TNF-α production are severely impaired in peritoneal macrophages isolated from mice carrying heterozygous disruption of the SOCS-3 gene. Finally, we show that SOCS-3 constitutive expression almost completely blocks IL-10-induced STAT3 tyrosine phosphorylation. Collectively, our data suggest that SOCS-3 may act as a negative feedback regulator and as an intracellular mediator used by IL-10 to exert its anti-inflammatory actions.

Materials and Methods

Cell culture and stable transfection

The mouse macrophage cell line J774 (provided by Dr. V. Kruys, Université Libre de Brussels, Brussels, Belgium) was maintained in DMEM (Bio-Whittaker, Verviers, Belgium) supplemented with 5% low endotoxin FBS (Biochrom Seromed, Berlin, Germany), and passaged twice weekly. Cells (8×10^5 cells/well) were seeded into six-well plates and transfected 24 h later with the pcDNA3-*myc*-SOCS-3 construct (17) using the Superfect transfection reagent (Qiagen, Valencia, CA), according to the manufacturer's instructions. Stably transfected cells were selected 3 days after transfection by addition of 0.3 mg/ml Geneticin (Life Technologies, Paisley, U.K.). These concentrations were maintained for the first 3 days and increased every third day up to 0.7 mg/ml. Twenty-four clones were then isolated and cultured in the presence of 0.7 mg/ml Geneticin. In all the experiments cells were seeded in tissue culture plates and stimulated 24 h later with 100 ng/ml LPS (from *Escherichia coli* serotype 026:B6; Sigma-Aldrich, St. Louis, MO) in the presence or the absence of 20 ng/ml murine IL-10 (Immunokontact, Abingdon, U.K.). In selected experiments cell were also stimulated with 10 ng/ml murine IFN-γ (PeproTech, London, U.K.) or 10 ng/ml murine IL-6 (Immunokontact). All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use.

Primary mouse macrophages isolation and culture

Primary resident macrophages were isolated by peritoneal lavages with 10 ml RPMI 1640 containing 10% FBS from 6- to 8-wk-old SOCS3$^{+/-}$ mice (18) backcrossed onto a C57BL/6 background and from their wild-type (SOCS3$^{+/+}$) littermates. Cells were seeded at a density of $\sim 1 \times 10^6$ cells/well in 24-well tissue culture plates and were cultured overnight in RPMI 1640 plus 10% FBS at 37°C in a humidified incubator containing 5% CO$_2$. The following day, all nonadherent cells were removed by washing with PBS. Adherent primary macrophages were then stimulated with LPS (100 ng/ml) in the presence or the absence of 20 ng/ml IL-10 for 3–12 h, and the production of NO and TNF-α was determined as described below.

Total RNA extraction and RNase protection assay (RPA) analysis

After stimulation with LPS and/or IL-10 or IFN-γ, cells were harvested, and total RNA was extracted using a commercial kit (SV total RNA isolation system; Promega, Madison, WI). Analysis of the expression of different genes was conducted at once by RPA, using the RiboQuant Custom Mouse Probe Set containing probes for iNOS, TNF-α, IL-1Ra, IL-6, L32, and GAPDH (BD Pharmingen, La Jolla, CA) according to the manufacturer's instructions. Expression levels of the various genes were quantified by Instantimager analysis (Packard Instruments, Palo Alto, CA).

Western blots

Preparation of cell lysates and immunoblot analysis were conducted as previously described (19) using the following primary Abs: anti-NH$_2$ terminus SOCS-3 (Immuno-Biological Laboratories, Tokyo, Japan) diluted at 5 μg/ml; anti-Y$_{705}$-P STAT3, anti-T$_{202}$/Y$_{204}$-P extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK), anti-T$_{180}$/Y$_{182}$-P p38 MAPK, anti-T$_{183}$/Y$_{185}$-P stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) MAPK, anti-p38 MAPK, and anti-SAPK/JNK MAPK (New England Biolabs, Beverly, MA) diluted as recommended by the manufacturer; and anti-STAT3 diluted at 1/2000, anti-ERK1 and anti-ERK2 diluted 1/1000 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-iNOS (Transduction Laboratories, Lexington, KY) diluted at 1/500. Ab binding was detected by using HRP-conjugated anti-mouse or anti-rabbit IgG (1/4000 dilution in TBS-Tween) and was revealed using the chemiluminescence system (ECL; Amersham Pharmacia Biotech, Arlington Heights, IL).

Detection of cytokine and nitrite secretion

The extracellular release of cytokines and nitrite was assayed in cell-free supernatants collected after cell stimulation for the indicated times. Cytokine levels in cell-free supernatants of J774 cells and primary macrophages were determined using commercial ELISA kits for mouse TNF-α (Minikit; Endogen, Woburn, MA), mouse GM-CSF (Quantikine Immunoassay; R&D Systems, Minneapolis, MN), and mouse IL-6 (Quantikine). The production of NO was determined by assaying culture supernatants for NO$_2^-$ accumulation by the Griess reagents (20).

Statistical analysis

Data are expressed as the mean $\pm$ SE. Statistical evaluation was performed using Student's t test and was considered significant at $p < 0.05$.

Results

### Stable expression of SOCS-3 in the J774 mouse macrophage cell line

To clarify whether IL-10-induced SOCS-3 acts as a key mediator of IL-10 anti-inflammatory responses, we stably transfected Myc-tagged SOCS-3 cDNA into the mouse macrophage cell line J774. Our preliminary experiments demonstrated that in these cells IL-10 not only strongly inhibits LPS-induced NO and TNF-α production, but also induces SOCS-3 mRNA and protein expression. Three weeks after antibiotic selection, transfectants were cloned, and expression levels of SOCS-3 in individual clones (named JS) were verified by immunoblot analysis, using anti-SOCS-3 Abs. Albeit at variable levels, all the clones isolated constitutively expressed a protein of the predicted molecular mass of Myc-SOCS-3, but only four of them (JS#10, JS#11, JS#L, and JS#13) displayed levels of exogenous *myc*-SOCS-3 expression similar to those of endogenous SOCS-3 induced by IL-10 plus LPS in parental J774 cells (Fig. 1). We therefore chose these four clones for subsequent analysis of LPS responsiveness.

Effect of SOCS-3 on LPS-induced iNOS expression and NO production

Fig. 2 shows that in J774 macrophages LPS-induced iNOS mRNA accumulation is detectable as early as after 2 h of incubation, peaks at 6 h, and gradually decreases over 24 h (not shown). In the presence of IL-10, LPS-induced iNOS mRNA expression is inhibited by 45% after 2 h and by 69% after 6 h. Similarly, the levels of iNOS mRNA induced by LPS in SOCS-3-expressing clones resulted lower compared with those in LPS-stimulated J774 parental cells. The degree of inhibition of LPS-induced iNOS mRNA expression in the different clones was variable, corresponding at
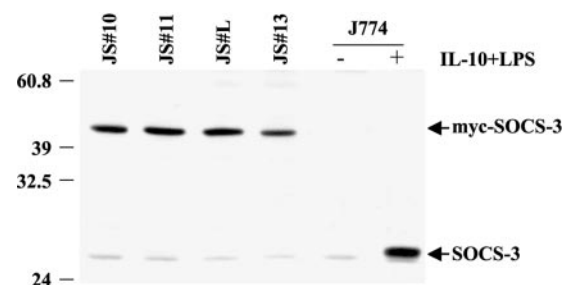
FIGURE 1. Expression of SOCS-3 in stably transfected JS clones. SOCS-3-transfected clones (JS#10, JS#11, JS#L, JS#13) and wild-type J774 incubated for 6 h with IL-10 in combination with LPS, were lysed as described in *Materials and Methods*. Fifty micrograms of whole cell extracts were electrophoresed, blotted, and analyzed for SOCS-3 protein expression using a specific anti-NH$_2$ terminus SOCS-3 Ab. The arrows indicate endogenous SOCS-3 and exogenous Myc-tagged SOCS-3 proteins, respectively. The experiment shown is representative of three.
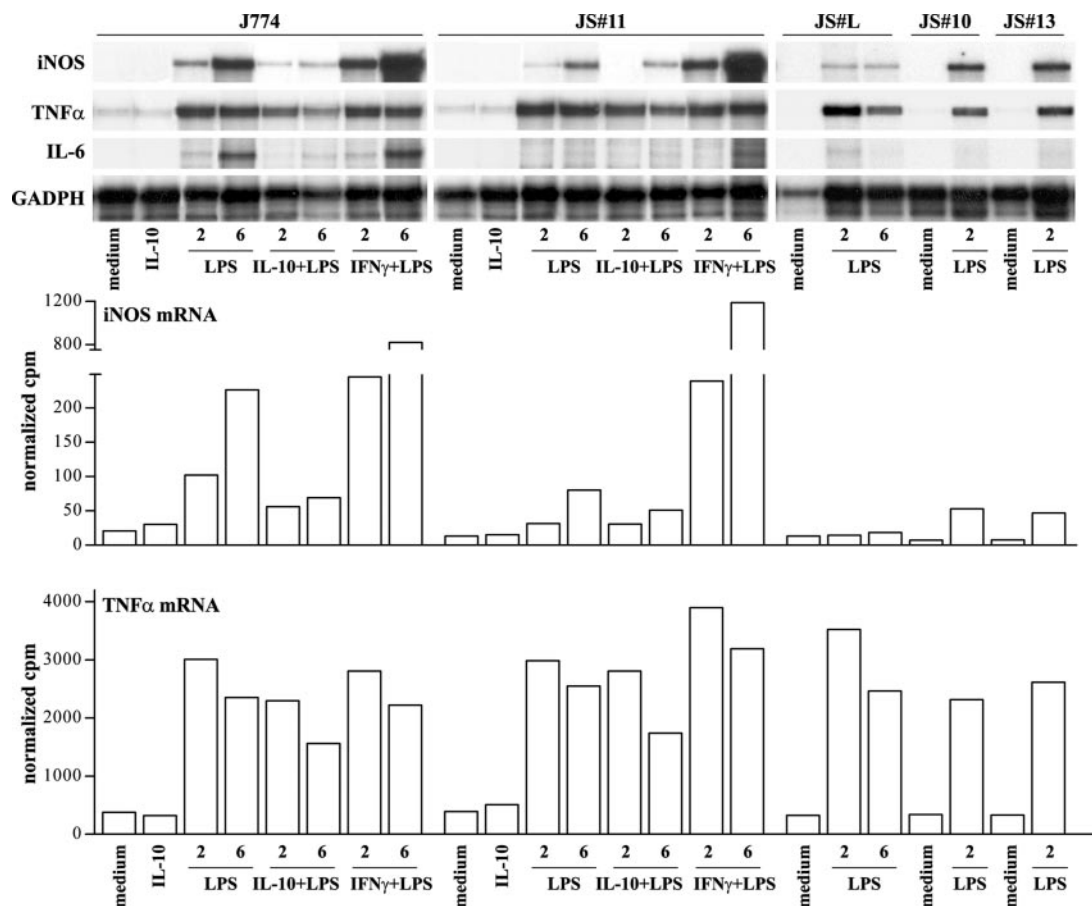
FIGURE 2. Modulation of LPS-induced proinflammatory gene expression by constitutively expressed SOCS-3. Parental J774 and SOCS-3-transfected clones (JS#11, JS#L, JS#10, JS#13) were stimulated for the indicated times with 100 ng/ml LPS, alone or in combination with 20 ng/ml IL-10 or 10 ng/ml IFN-γ. Total RNA was isolated and subjected to RPA analysis as described in *Materials and Methods*. The amount of radioactivity for each condition was then quantified using an Instant Imager and normalized to the corresponding GAPDH expression. The *top panel* shows the mRNA expression of iNOS, TNF-α, IL-6, and GAPDH, whereas the *bottom panels* report a quantitative representation for iNOS and TNF-α mRNAs. This experiment is representative of four performed.

2 h to 69% in JS#11, 85% in JS#L, 48% in JS#10, and 54% in JS#13. In contrast, iNOS mRNA steady state levels induced by LPS plus IFN-γ was not modified in SOCS-3-expressing clones compared with parental J774.

To verify whether SOCS-3 transfection also inhibited iNOS protein expression in LPS-treated cells, whole cell lysates of parental J774 incubated with LPS, alone or in combination with IL-10, for 3, 6, and 24 h, as well as cell lysates of JS#11 and JS#13 clones incubated with LPS alone were subjected to Western blot analysis with anti-iNOS Abs (Fig. 3A). Under these conditions, LPS-induced iNOS protein in J774 cells was already detectable after 3 h and continued to accumulate up to 24 h, but was strongly diminished if IL-10 was present in the culture medium (Fig. 3A). Similarly, and in line with RPA analysis, the synthesis of iNOS protein induced by LPS in JS clones was lower than that induced in J774 (Fig. 3A). As a result of the reduced levels of LPS-induced iNOS enzyme observed either in J774 treated with IL-10 or in SOCS-3-expressing JS clones, the production of NO was, accordingly, decreased. Addition of IL-10 to parental J774 in fact reduced LPS-induced NO production by an average of $61 \pm 5\%$ ($n = 6$) at 24 h. Similarly, NO production in response to LPS was reduced in JS clones compared with control J774 by an average of $82 \pm 4\%$ in clone JS#11 ($n = 8$), $51 \pm 7\%$ in clone JS#13 ($n = 2$), and $88 \pm 7\%$ in clone JS#L ($n = 5$; Fig. 3B). However, NO production triggered by IFN-γ in combination with LPS was not significantly inhibited by either IL-10 in parental J774 (not shown) or forced SOCS-3 expression (Fig. 3B).

Effect of SOCS-3 on LPS-induced TNF-α expression and production

Because IL-10 is well known to suppress cytokine production in many cellular systems, including J774 (21, 22), in subsequent experiments we investigated the effect of forced SOCS-3 expression on LPS-induced TNF-α. In agreement with previous studies, addition of IL-10 resulted in a strong inhibition of LPS-induced TNF-α production in J774 cells (Fig. 4A), which was, on the average, $48 \pm 10\%$ at 3 h, $80 \pm 8\%$ at 6 h, and $80 \pm 13\%$ at 24 h ($n = 4$; Fig. 4B). Similarly, stable expression of SOCS-3 reduced TNF-α production in response to LPS by an average of $70 \pm 15\%$ at 3 h, $85 \pm 5\%$ at 6 h, and $52 \pm 18\%$ at 24 h in clone JS#11 ($n = 4$) and by an average of $88 \pm 6\%$ at 6 h and $77 \pm 4\%$ at 24 h in clone JS#10 ($n = 5$; Fig. 4, A and B).

To determine whether inhibition of TNF-α production by activated JS clones reflected a poor induction of TNF-α transcripts by LPS, we quantified the amounts of TNF-α mRNA following LPS stimulation in the presence or the absence of IL-10 in parental J774 cells or in SOCS-3-expressing clones. As shown in Fig. 2, TNF-α mRNA accumulation was rapidly induced by LPS in wild-type J774, peaking at 2 h and slightly decreasing within 6 h. Differently

FIGURE 3. Constitutively expressed SOCS-3 inhibits LPS-induced iNOS protein expression and NO production. *A*, J774, JS#11, and JS#13 stimulated with LPS alone or in combination with IL-10 were lysed at the indicated times. Cellular extracts (10 μg/lane) were then loaded on the gels, and immunoblots were performed using specific anti-iNOS Abs. Results are from one experiment representative of two performed. *B*, J774 and JS clones (10^5 cells/well) were cultured in the presence of LPS with or without IL-10 or IFN-γ for 24 h. NO_2^- concentrations in cell-free supernatants were determined by the Griess reaction. The mean $\pm$ SE are shown, and data were obtained from six experiments in J774, eight in JS#11, two in JS#13, and five in JS#L.
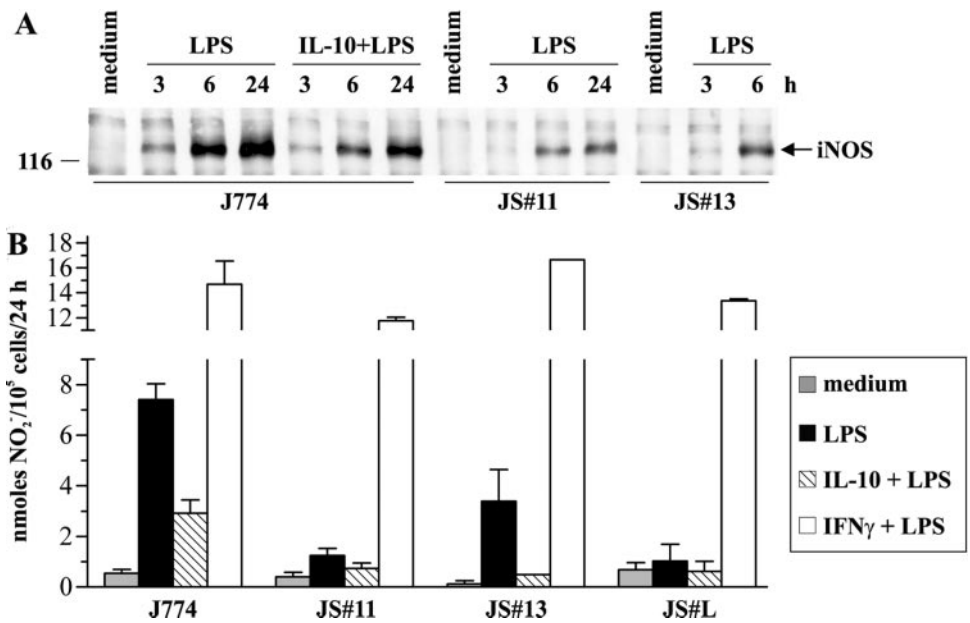
from the effect on iNOS mRNA induction, IL-10 inhibited LPS-induced TNF-α mRNA accumulation by only $17 \pm 8\%$ at 2 h and $29 \pm 11\%$ at 6 h (Fig. 4*B*, average of three independent experiments). In a similar manner the levels of LPS-induced TNF-α transcripts in clones JS#11 and JS#L were not significantly different from those observed in parental J774 cells (Fig. 2) and were only slightly reduced in clones JS#10 (23% inhibition at 2 h) and JS#13 (13% inhibition at 2 h; Figs. 2 and 4*B*). Overall, these data indicate that IL-10 as well as SOCS-3 constitutive expression inhibit macrophage production of TNF-α primarily at a post-transcriptional level.

Effect of SOCS-3 on LPS-induced IL-6 and GM-CSF production

Analysis of the effects of SOCS-3 forced expression was then extended to LPS-induced IL-6 gene expression and protein secretion as well as to LPS-induced GM-CSF production. As shown in the RPA of Fig. 2, up-regulation of IL-6 mRNA by LPS peaked at 6 h and was strongly suppressed by IL-10. In a similar manner LPS failed to induce IL-6 gene expression in SOCS-3-expressing clones (Fig. 2). In contrast with iNOS and TNF-α, IL-6 transcripts

induced by LPS plus IFN-γ was inhibited in SOCS-3-expressing clones, indicating that the expression of IL-6 mRNA is regulated differently from that of iNOS or TNF-α. In line with the results obtained at the mRNA level, the production of IL-6 induced by LPS was dramatically inhibited by IL-10 in J774 cells and by constitutive expression of SOCS-3 in JS clones (Table I). Under the same experimental conditions the production of GM-CSF stimulated by LPS was strongly inhibited by IL-10 in J774 and was totally blocked in JS clones (Table I).

Effect of SOCS-3 on IL-1ra mRNA accumulation

IL-10 exerts its anti-inflammatory actions not only by inhibiting the production of proinflammatory mediators, but also by promoting the synthesis and release of natural inhibitors of certain proinflammatory cytokines (8). According to previous findings (23), Fig. 5 shows that in parental J774 cells the IL-1ra gene is strongly up-regulated in response to LPS and is further enhanced in cells costimulated with IL-10. Remarkably, RPA analysis demonstrates that there is a slight increase in the basal levels of IL-1ra gene expression in all JS clones (Fig. 5). However, in contrast with the
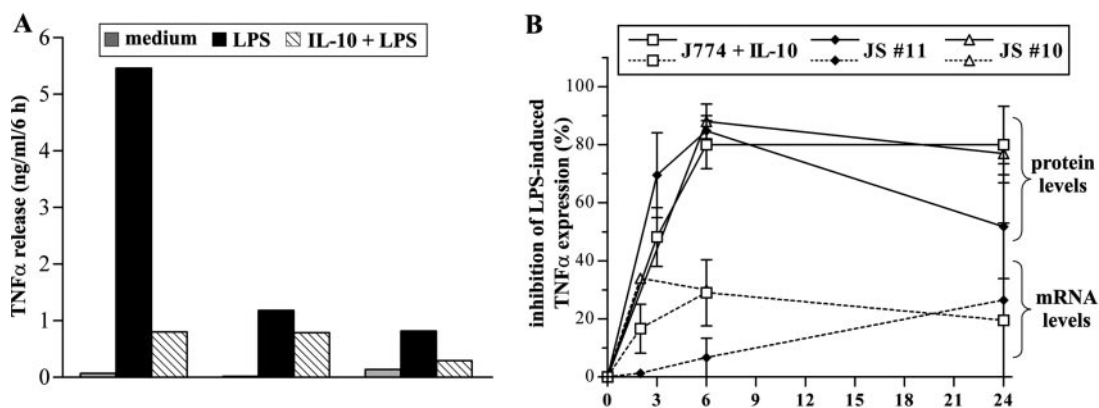
FIGURE 4. Constitutively expressed SOCS-3 inhibits LPS-induced TNF-α secretion. *A*, J774, JS#11, and JS#10 were stimulated for 6 h with LPS alone or in combination with IL-10. Cell-free supernatants were collected, and extracellular TNF-α secretion was quantified by ELISA. A representative experiment of four performed with similar results is shown. *B*, The data are the percent inhibition of LPS-induced TNF-α mRNA expression (dotted lines) and protein secretion (continuous lines) by either IL-10 in J774 cells ($\square$) or constitutive SOCS-3 expression in JS#11 ($\blacklozenge$) and JS#13 ($\triangle$). Values are the mean $\pm$ SE of four independent experiments for protein secretion and three experiments for mRNA expression.

Table I. *Inhibition of LPS-induced cytokine production (%)[a]*

	IL-6	GM-CSF
J774 + IL-10	65.8 ± 21 (2)	96.9 ± 0.8 (2)
JS#11	100 (2)	96.9 ± 2.2 (5)
JS#L	100 (1)	98.3 ± 1.7 (2)
JS#13	Nd	68.8 (1)

[a] Cell free supernatants of J774 stimulated with LPS with or without IL-10 and of JS clones stimulated with LPS were collected at 24 h. The amounts of secreted GM-CSF and IL-6 were quantified using specific ELISAs. The values indicate the percentage of inhibition (means ± SE) of LPS-induced IL-6 and GM-CSF production. The number of experiments is indicated in parentheses.

inhibitory effect of SOCS-3 forced expression on endotoxin stimulation of proinflammatory mediators, addition of LPS was still effective in increasing IL-1ra mRNA expression in JS clones.

Effects of IL-10 on various signaling pathways triggered by LPS

Activation of ERK1/2, SAPK/JNK, and p38 MAPK as well as NF-κB has been reported to be involved in the signaling cascade(s) mediating LPS-induced TNF-α production (24). To investigate whether IL-10 treatment or SOCS-3 constitutive expression inhibits LPS-induced TNF-α production by interfering with MAPK/SAPK signaling, we examined the effects of IL-10 and SOCS-3 transfection on the LPS-induced activation of ERK1/2, SAPK/JNK, and p38 MAPK using phospho-specific Abs (Fig. 6). Fig. 6A shows that maximal activation of p38 by LPS is achieved within 2 min, remains stable over 15 min, and decreases thereafter (not shown), whereas optimal activation of ERK1/2 and SAPK/JNK in wild-type J774 is achieved after 30 min of LPS stimulation and remains unchanged at 60 min (Fig. 6B). In these experiments neither IL-10 in wild-type J774 (Fig. 6, A and B) nor SOCS-3 expression in JS clones #10 (Fig. 6, A and B) and #11 (not shown) influenced either the levels or the kinetics of p38 MAPK, ERK1/2, and SAPK/JNK activation in LPS-stimulated cells. The apparent decrease in SAPK/JNK phosphorylation in J774 in the presence of IL-10 and in JS clones was due to the slightly reduced levels of SAPK/JNK proteins in these cells (Fig. 6B). Similarly, LPS-induced NF-κB activation was unaffected by either IL-10 or SOCS-3 expression in J774 cells (data not shown).
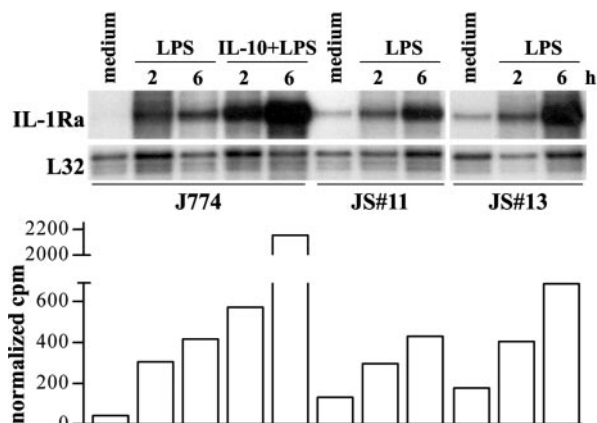
FIGURE 5. Consitutive expression of IL-1ra in SOCS-3-expressing cells. Total RNA was extracted from J774 and JS clones stimulated with LPS and/or IL-10 plus LPS for the indicated times and processed for RPA analysis. The *top panel* shows mRNA expression of IL-1ra and the L32 housekeeping gene, whereas the *bottom panel* reports a quantitative representation of IL-1ra gene expression, after normalization. The experiment shown is representative of three performed.
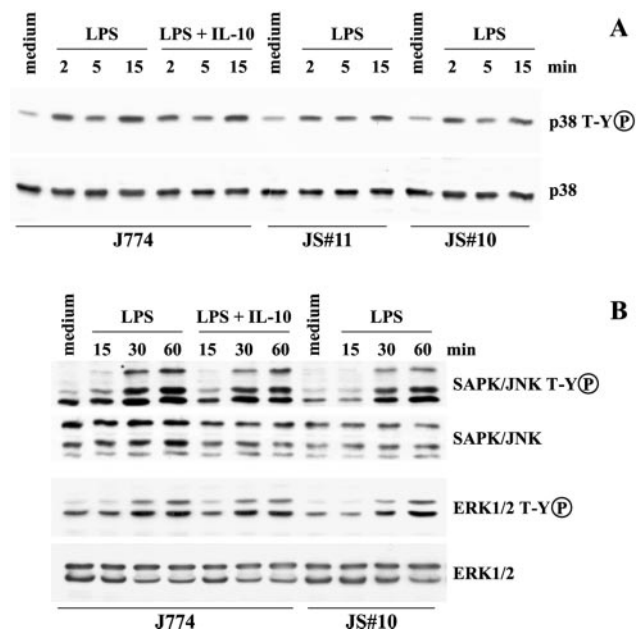

FIGURE 6. LPS-induced MAPK activation is not modified by IL-10 treatment or SOCS-3 expression. *A*, J774, and JS#11, and JS#10 clones were stimulated for 2, 5, and 15 min with LPS alone or in combination with IL-10. An immunoblot of whole cell lysates (40 μg/lane) with anti-Y-T-P p38 MAPK Ab (*upper panel*) or anti-p38 MAPK Ab (*lower panel*) is shown. *B*, J774 and JS#10 were incubated for 15, 30, and 60 min with LPS or IL-10 plus LPS. Immunoblotting of whole cell extracts (40 μg/lane) was performed with anti-Y-T-P SAPK/JNK (SAPK/JNK T-Y P) and anti-T-Y-P ERK 1/2 (ERK1/2 T-Y P). Membranes were stripped and reprobed for detection of the total protein content of each kinase. The blots shown are representative of three independent experiments.

Decreased ability of IL-10 to regulate LPS-responsiveness in SOCS-3^{+/−} macrophage

To provide more evidence on the capacity of SOCS-3 to mediate the inhibitory effects of IL-10, we monitored whether IL-10 was able to inhibit LPS-induced TNF-α and NO production in primary peritoneal macrophages isolated from SOCS-3^{+/−} mice (18) backcrossed onto a C57BL/6 background and from their wild-type (SOCS-3^{+/+}) littermates. As depicted in Fig. 7, *A* and *B*, showing representative experiments, IL-10 inhibited the production of TNF-α and the release of NO from LPS-activated macrophages harvested from wild-type mice by 69.7 ± 8.9% (*n* = 3; Fig. 7A) and 71.5 ± 2.8% (*n* = 3; Fig. 7B), respectively. In contrast, the ability of IL-10 to inhibit LPS-induced TNF-α and NO production in macrophages derived from SOCS-3^{+/−} mice decreased to 20.3 ± 3.7% (Fig. 7A), and to 31 ± 0.9% (Fig. 7B), respectively. The reduced inhibitory activity of IL-10 on LPS-induced TNF-α and NO production in SOCS-3^{+/−} cells is significantly different from that in SOCS-3^{+/+} cells (*p* = 0.001). In parallel, Western blot analysis revealed that LPS plus IL-10-induced SOCS-3 protein expression was substantially reduced in adherent macrophages from SOCS-3^{+/−} mice compared with macrophages from SOCS-3^{+/+} mice (Fig. 7C), indicating that SOCS-3 is required for mediating the anti-inflammatory action of IL-10.
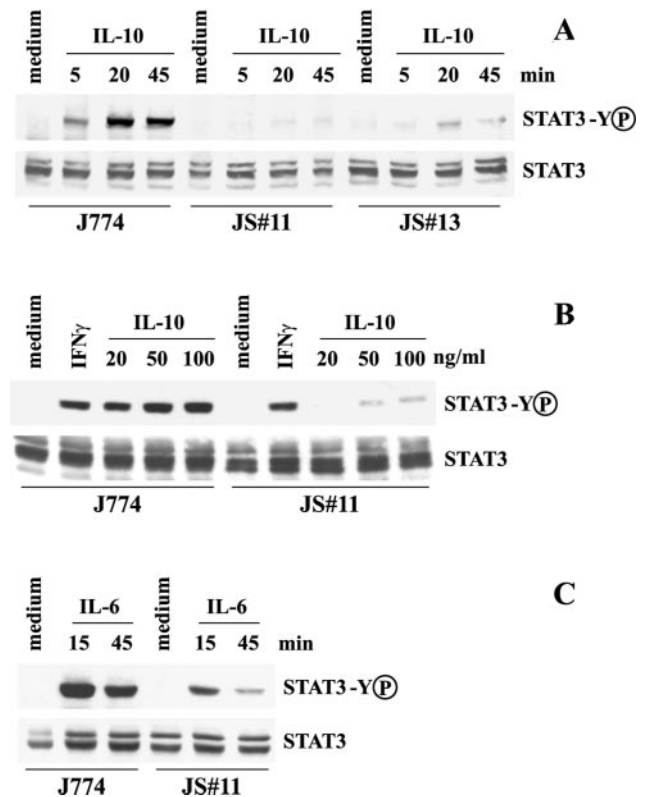
Effect of SOCS-3 on IL-10 signaling

The activation of STAT3 has been shown to play a key role in IL-10 signal transduction (8). In contrast, SOCS-3 has been described to target the STAT pathway activated by different cytokines (14). We therefore examined whether activation of STAT3 in response to IL-10 was impaired in SOCS-3-expressing clones. As
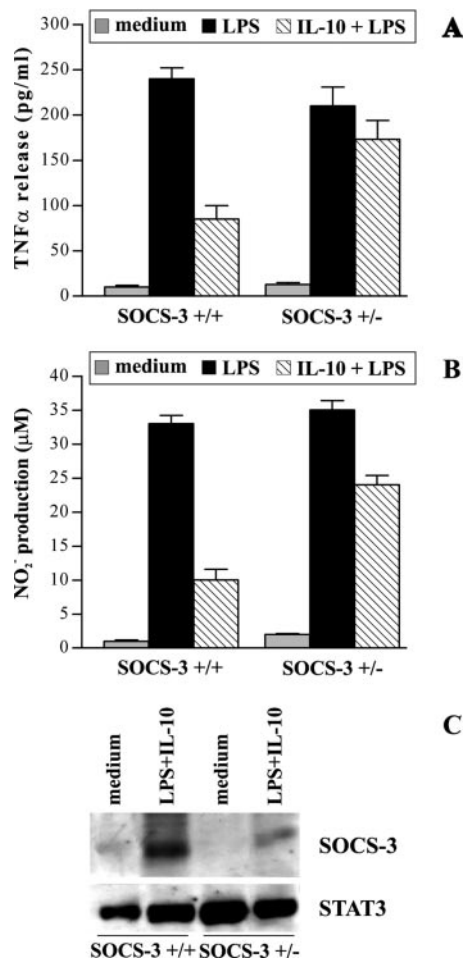
FIGURE 7. Defective IL-10 inhibitory actions in SOCS-3$^{+/-}$ macrophages. Primary peritoneal macrophages isolated from wild-type and SOCS-3$^{+/-}$ mice were collected and plated as described in *Materials and Methods* and were stimulated with LPS (100 ng/ml) alone or in combination with IL-10 (20 ng/ml) for 12 h. *A* and *B*, TNF-α levels in supernatants were quantified via ELISA. NO$_2^-$ secretion was detected by Griess method. *C*, Whole cell lysates were immunoblotted with anti-SOCS-3 Ab (*upper panel*). Equal amounts of total protein loading were confirmed by subsequent reprobing with anti-STAT3 Ab (*lower panel*). The mean ± SD of duplicate data are shown and are representative of three experiments with cells derived from individual mice.

FIGURE 8. Constitutive SOCS-3 expression inhibits IL-10-induced STAT3 tyrosine phosphorylation. J774 and JS clones were stimulated for 5, 15, and 45 min with 20 ng/ml IL-10 (*A*); for 15 min with 20, 50, and 100 ng/ml IL-10 or 10 ng/ml IFN-γ (*B*), or for 15 and 45 min with IL-6 (*C*). The *upper panels* show an immunoblot of whole cell lysates (50 μg/lane) performed with anti-Y$_{705}$-P STAT3 Abs. The *lower panels* represent the same membranes reprobed with anti-STAT3 to ensure that similar amounts of protein were present in each lane. The results shown are representative of three independent experiments.

shown in Fig. 8*A*, IL-10 promotes a strong and time-dependent STAT3 tyrosine phosphorylation in J774 cells that is, however, strongly inhibited in JS#11 and JS#13 cells (Fig. 8*A*), even if the cytokine is used at doses up to 100 ng/ml (Fig. 8*B*). STAT3 tyrosine phosphorylation induced by IL-6 was also substantially inhibited by SOCS-3 constitutive expression (Fig. 8*C*), confirming the results obtained in other SOCS-3-transfected cells (25–30). In contrast, tyrosine phosphorylation of STAT3 in response to IFN-γ was not influenced by the presence of SOCS-3 (Fig. 8*B*), excluding that the lack of responsiveness to IL-10 or IL-6 was due to a general inability to phosphorylate STAT3 on tyrosine residues by activated JS clones. Consistent with these results, IL-10- and IL-6-induced DNA-binding activities to the oligonucleotide probe hSIE/m67 were severely reduced in JS clones (data not shown).

Finally, we asked whether inhibition of IL-10-induced STAT3 activation by the constitutive expression of SOCS-3 would cause the loss of some biological responses triggered by IL-10. For this purpose, we monitored the ability of IL-10 to inhibit LPS-induced NO and TNF-α production in JS clones. As shown in Figs. 3*B* and

4*A*, although NO and TNF-α production triggered by LPS treatment was already significantly inhibited by forced SOCS-3 expression, IL-10 was able to further suppress these functions in JS clones. Treatment of J774 and JS clones with IL-10 in fact inhibited LPS-induced NO at 24 h by an average of 61 ± 5% in J774 ($n = 6$; $p < 0.001$), 36 ± 16% in JS#11 ($n = 4$; $p < 0.05$), 81 ± 4% in JS#13 ($n = 2$; $p < 0.05$), and 69 ± 16% in JS#L ($n = 3$; $p < 0.05$), and LPS-induced TNF-α at 6 h by an average of 80 ± 16% in J774 ($n = 4$; $p < 0.005$), 37 ± 10% in JS#11 ($n = 4$; $p < 0.005$), and 67 ± 4% in JS#10 ($n = 2$; $p \leq 0.01$).

Discussion

The focus of this work was to study the mechanisms that underlie the ability of IL-10 to inhibit the production of proinflammatory mediators by LPS-stimulated phagocytes. Several data have pointed out that the capacity of IL-10 to inhibit monocyte and neutrophil activation by LPS can be blocked by the protein synthesis inhibitor cycloheximide (10, 11). Recently, it has been reported that IL-10 directly stimulates the expression of SOCS-3 mRNA and protein in both human monocytes and neutrophils (12, 13, 19). Due to the ability of SOCS-3 to negatively regulate the responses to different activating cytokines and bacterial products, it has been speculated that IL-10 exerts its anti-inflammatory action by inducing SOCS-3 expression (16). However, direct evidence that SOCS-3 can act as one of the mediators of IL-10 inhibitory action on LPS-induced macrophage activation has never

been provided. Furthermore, whether IL-10-induced SOCS-3 also provides a negative feedback regulation of IL-10 signaling itself has never been determined. To clarify these issues, we studied the effect of SOCS-3 forced expression in the J774 macrophage cell line, which has been well characterized for its responsiveness to IL-10 (21).

Our data show that SOCS-3, stably transfected in the J774 cell line at levels comparable to those of endogenous SOCS-3 induced in response to IL-10 plus LPS in parental cells, inhibited LPS-induced production of several proinflammatory mediators, including TNF-α, IL-6, GM-CSF, and NO. SOCS-3 transfection did not, however, influence TNF-α or NO induction stimulated by LPS used in combination with IFN-γ, thereby demonstrating that its effects were selective. SOCS-3-mediated inhibitory effects were quantitatively comparable to the degree of inhibition exerted by IL-10 on LPS-stimulated wild-type cells and were achieved by molecular mechanisms acting at both transcriptional and post-transcriptional levels that resembled those used by IL-10 (10, 11, 31–33). For instance, we found that while LPS-induced TNF-α production was dramatically inhibited in JS clones or by IL-10 in J774 cells, inhibition of LPS-induced TNF-α mRNA accumulation was marginal. Although we cannot exclude regulation at the level of TNF-α secretion, our data support the hypothesis recently proposed by Kontoyiannis and colleagues that IL-10-activated SOCS-3 might block the signaling pathway(s) required to activate post-transcriptional mechanisms regulating TNF-α synthesis (34). LPS-induced activation of MAPK/SAPK such as ERK, JNK, and p38, has been shown to regulate TNF-α gene expression at the level of translation (35–37). In this context, whether IL-10 exerts its inhibitory action by interfering with the LPS-induced activation of ERK, JNK, or p38 is not clear, since the results reported in the literature are contradictory (38–42). The data presented in this study, showing that LPS-induced activation of ERK1/2, SAPK/JNK, and p38 MAPK remain unaltered following IL-10 addition in wild-type J774, are in line with other work reporting similar observations (16, 43, 44). Similarly, we show that in JS clones also the activation of the various MAPK/SAPK signaling pathways by LPS is unaffected by SOCS-3, adding further evidence that IL-10 and SOCS-3 actions proceed in parallel.

In contrast with the effects of IL-10 and SOCS-3 on TNF-α expression and production, we consistently observed a correlation between the decrease in iNOS mRNA levels and the suppression of NO production in parental J774 cells treated with LPS in the presence of IL-10 as well as in JS clones after LPS stimulation. The data confirm the inhibitory effect of SOCS-3 constitutive expression on LPS-dependent NO synthesis in the murine M1 myeloid cell line previously reported (13). Moreover, either IL-10 treatment or SOCS-3 forced expression in LPS-activated J774 caused the inhibition of IL-6 mRNA expression and protein secretion. This is in contrast with the result obtained in M1 cell line, in which SOCS-3 forced expression did not affect LPS-induced expression of IL-6 mRNA (13). However, we have recently observed that in those M1-trasfected cells, cytoplasmic *myc*-SOCS-3 is expressed at lower levels than those detected in JS clones (C. Berlato, A. Yoshimura, unpublished observations). We would therefore tend to speculate that in M1 cells the amount of transfected SOCS-3 is not enough to prevent LPS-induced IL-6 gene expression.

Another novel and very interesting observation uncovered by our study is that cells expressing SOCS-3, unlike parental J774, constitutively accumulate IL-1ra mRNA transcripts. Addition of LPS to JS clones further enhanced IL-1ra mRNA expression, indicating first that LPS responses are not generally inhibited in JS clones, and secondly that SOCS-3 constitutive expression also reproduces the IL-10-enhancing properties on LPS-mediated induc-

tion of the IL-1ra gene (8, 23). Whether SOCS-3 induces expression of IL-1ra mRNA directly via transcription factor activation or through a positive modulation of IL-1ra mRNA stability, similar to the effect of IL-10 in LPS-stimulated neutrophils (23), remains to be established. Nonetheless, in consideration of the anti-inflammatory effects of IL-1ra, these data further support an involvement of SOCS-3 in those mechanisms by which IL-10 exerts its immunosuppressive actions by up-regulating anti-inflammatory molecules.

In addition to the findings that SOCS-3 constitutive expression inhibits LPS-induced proinflammatory responses in J774 cells, we demonstrate that SOCS-3 is required for the IL-10-mediated deactivation of primary peritoneal macrophages treated with LPS. SOCS-3-null macrophages are not available, since homozygous disruption of the SOCS-3 gene leads to embryonic lethality as a consequence of erythrocytosis or abnormal placental development (18, 31). We therefore took advantage of mice carrying heterozygous disruption of the SOCS-3 gene, which are phenotypically normal and fertile (18). We observed that in primary peritoneal SOCS-3$^{+/-}$ macrophages the induction of SOCS-3 protein expression in response to LPS plus IL-10 was severely impaired. In parallel with such reduced induction of cytoplasmic SOCS-3, the inhibitory activity of IL-10 on LPS-induced TNF-α and NO production was significantly reduced, clearly indicating that SOCS-3 is involved in the negative signaling pathways triggered by IL-10.

Taken together, our observations represent the first evidence that SOCS-3 induced by IL-10 takes part in the inhibitory pathways activated by this cytokine and point out for a broad role for SOCS-3 protein in regulating macrophage deactivation by inhibiting the production of proinflammatory signals and by favoring the expression of anti-inflammatory molecules as well. Future work, aimed at understanding the molecular mechanism(s) by which SOCS-3 mediates suppression of the LPS signaling pathway, will certainly help in understanding the intracellular mechanisms by which IL-10 inhibits proinflammatory cytokine production by LPS-activated phagocytes. In regard to the latter issue, it has been reported that IL-10 down-regulates the expression of TLR4, the signal transducing receptor for LPS (32). However, because induction of TNF-α, IL-1ra, and macrophage inflammatory protein-2 (not shown) mRNAs in response to LPS was not significantly impaired in JS clones compared with J774 parental cells, we would exclude the possibility that the impairment of LPS responses observed in SOCS-3-transfected cells was a consequence of a decreased expression of TLR4.

In this study we also report, for the first time, that STAT3 tyrosine phosphorylation in response to IL-10 and STAT3 DNA-binding activities as well are severely impaired in SOCS-3-transfected cells. A critical role for STAT3 in mediating IL-10-induced inhibition has been demonstrated by targeted deletion of the STAT3 gene in macrophages and neutrophils (33). However, it has also been demonstrated that STAT3 activation is not sufficient to mediate the anti-inflammatory action of IL-10 (34, 40). In our experiments we found that SOCS-3 constitutive expression, despite its inhibitory effect on IL-10-induced STAT3 tyrosine phosphorylation, did not completely abolish the down-regulatory activities of IL-10 on LPS-induced NO and TNF-α production. Although we cannot exclude that the minimal tyrosine phosphorylation of STAT3 induced by IL-10 in JS clones was sufficient to mediate the residual inhibitory effects of IL-10, as a whole our data support the view that IL-10-mediated inhibition of TNF-α and NO production occurs through STAT3-dependent and STAT3-independent pathways (21). However, we cannot rule out the possibility that SOCS-3 forced expression was not sufficient to completely block the capacity of IL-10 to activate STAT3. Whatever the case, our data support the view that that an accessory signaling pathway,

in addition to the Jak-STAT pathway, is required for expression of the anti-inflammatory actions of IL-10 (22).

Defects in IL-10 production or in macrophage STAT3 signaling molecule (5, 33) or forced expression of a dominant negative form of SOCS-3 in mice (30) lead to the development or the worsening of chronic inflammatory pathologies such as inflammatory bowel disease or colitis. A detailed dissection of the mechanisms responsible for SOCS-3 activation and the identification of SOCS-3 intracellular targets is therefore essential for preventing immune dysregulation and chronic inflammatory diseases.

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