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J Immunol 2003; 170:1383-1391; doi: 10.4049/jimmunol.170.3.1383
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Suppressor of Cytokine Signaling 1 Inhibits IL-10-Mediated Immune Responses

Yaozhong Ding,* Dongmei Chen,* Adel Tarcsafalvi,* Ruthie Su,* Lihui Qin,† and Jonathan S. Bromberg2*‡

IL-10 has proved to be a key cytokine in regulating inflammatory responses by controlling the production and function of various other cytokines. The suppressor of cytokine signaling (SOCS) gene products are a family of cytoplasmic molecules that are essential mediators for negatively regulating cytokine signaling. It has been previously shown that IL-10 induced SOCS3 expression and that forced constitutive expression of SOCS3 inhibits IL-10/STAT3 activation and LPS-induced macrophage activation. In this report, we show that, in addition to SOCS3 expression, IL-10 induces SOCS1 up-regulation in all cell lines tested, including Ba/F3 pro-B cells, MC/9 mast cells, M1 leukemia cells, U3A human fibroblasts, and primary mouse CD4+ T cells. Induction of SOCS molecules is dependent on STAT3 activation by IL-10R1. Cell lines constitutively overexpressing SOCS proteins demonstrated that SOCS1 and SOCS3, but not SOCS2, are able to partially inhibit IL-10-mediated STAT3 activation and proliferative responses. Pretreatment of M1 cells with IFN-γ resulted in SOCS1 induction and a reduction of IL-10-mediated STAT3 activation and cell growth inhibition. IL-10-induced SOCS is associated with the inhibition of IFN-γ signaling in various cell types, and this inhibition is independent of C-terminal serine residues of the IL-10R, previously shown to be required for other anti-inflammatory responses. Thus, the present results show that both SOCS1 and SOCS3 are induced by IL-10 and may be important inhibitors of both IL-10 and IFN-γ signaling. IL-10-induced SOCS1 may directly inhibit IL-10 IFN-γ signaling, while inhibition of other proinflammatory cytokine responses may use additional IL-10R1-mediated mechanisms.


Interleukin-10 is a key regulatory molecule for suppressing immune responses by blocking the production and function of many cytokines by various cell types. It inhibits T cell production of IL-2 and IL-5; inhibits monocoyte and macrophage synthesis of IL-1α, IL-1β, IL-6, IL-8, IL-12, TNF-α, GM-CSF, and reactive oxygen and nitrogen intermediates (1–4); and suppresses IFN-γ synthesis in NK cells (5), dendritic cell-stimulated Th1 cells (6), and LPS-stimulated macrophages (7). Generally considered as a Th2 cytokine, IL-10 inhibits Th1-dominated responses such as contact hypersensitivity, rheumatoid arthritis, and inflammatory bowel disease in vivo, and has been used in the treatment of autoimmune and inflammatory diseases (8–13). IL-10 or IL-10R2 knockout mice have been shown to have highly polarized Th1 responses and develop a severe colitis, which supports the essential role of IL-10 in balancing the cytokine network (14, 15).

A family of molecules that is an important mediator of inhibitory signals has been recognized and described as the suppressors of cytokine signaling (SOCS)1 molecules (16). The family is characterized by an Src homology 2 (SH2) domain and a C-terminal, unique, conserved motif referred to as the SOCS box. There appear to be at least eight of these SOCS proteins (SOCS1 to SOCS7 and cytokine-inducible SH2 protein). SOCS1 has been shown to bind to all four Janus kinases (JAKs) through its central SH2 domain and to inhibit their kinase activity and thus their STAT activation. Investigation of SOCS1−/− mice has shown that SOCS1 is a critical inhibitor of IFN-γ signaling and function (17, 18). SOCS3 has also been shown to bind to JAKs and suppress cytokine signaling, but the mechanism by which SOCS3 inhibits cytokine-induced STAT activation is not completely understood. SOCS3 deletion results in embryonic lethality at 12–16 days, associated with marked erythrocytosis, indicating its essential role in the regulation of fetal liver erythropoiesis (19). Its lethality prevented testing of IFN-γ or IL-10 signaling in vivo, although SOCS3 overexpression in vitro can inhibit IFN-γ signaling (20). It has been shown that SOCS3, but not SOCS1, mRNA could be induced by IL-10 in human monocytes and polymorphonuclear neutrophils (PMN), and suggested that IL-10 inhibits IFN-γ-induced STAT1 activation through SOCS3 induction (21, 22). By forced expression of SOCS3 in macrophage cell line J774, it has further been shown that SOCS3 functions both as an LPS signal inhibitor and as a negative feedback regulator of IL-10/STAT3 signaling (23). However, whether IL-10 regulates the expression of other SOCS, the signaling requirement for SOCS3 induction, and their subsequent effects on cytokine signaling has not been elucidated.

In this study, we have further examined the regulation of SOCS molecules by IL-10 and found that, in addition to SOCS3 induction, the SOCS1 gene is also up-regulated in several different cell types and lineages. Up-regulation of SOCS mRNAs and inhibition of IFN-γ signaling are dependent on distal tyrosines (Y427 and Y477) of IL-10R1 and STAT3 phosphorylation, but appear independent of STAT1 or STAT5, and independent of IL-10R1 C-terminal serines that play a role in other anti-inflammatory responses (24). SOCS1

*Carl C. Icahn Institute for Gene Therapy and Molecular Medicine, †Department of Pathology, and 2Reca\(\text{\textregistered}\)anati/Miller Transplantation Institute, Mount Sinai School of Medicine, New York, NY 10029

Received for publication April 1, 2002. Accepted for publication November 20, 2002.

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1 This work was supported by National Institutes of Health Grant RO1 AI44929 and by the Baxter Extramural Grant Program (to J.S.B.).

2 Address correspondence and reprint requests to Dr. Jonathan S. Bromberg or Dr. Yaozhong Ding, Carl C. Icahn Institute for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1496, New York, NY 10029-6574. E-mail addresses: jon.bromberg@mountsinai.org or yaozhong.ding@mssm.edu

3 Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; SH2, Src homology 2; JAK, Janus kinase; PMN, polymorphonuclear neutrophil; m, murine; vIL-10, viral IL-10; bIL-10, human IL-10; EGFP, enhanced green fluorescence protein.

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0022-1767/03/S02.00

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induction by IFN-γ, or SOCS1 or SOCS3 overexpression, can negatively regulate IL-10 function and IL-10R1 signaling. These results indicate complex mechanisms of IL-10-mediated inhibition of IFN-γ signaling and inhibition of proinflammatory cytokine synthesis. These data suggest that SOCS are involved in negatively regulating IL-10 signaling, and IL-10 suppression of IFN-γ function might be directly through induction of SOCS1.

Materials and Methods

Cell lines

The mouse pro-B cell line Ba/F3 expressing recombinant series of constructs of murine (m)IL-10R1 was maintained in RPMI 1640 medium containing 10% FCS, 5 ng/ml mIL-3 (PeproTech, Rocky Hill, NJ), and 0.5 mg/ml G418 (Life Technologies, Grand Island, NY) (25, 26). The mouse mast cell line MC/9 was maintained in DMEM containing 10% FCS and 5% Con A supernatant prepared from CBA mouse spleen cells (26). The mouse leukemia M1 cell line was purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM containing 10% FCS, with 0.5 mg/ml G418 for mIL-10R1-transfected cells. The STAT1-deficient human fibroblast U3A cell line was kindly provided by Dr. G. Stark (The Cleveland Clinic Foundation, Cleveland, OH) and maintained as described previously (27, 28). Spleenic CD4+ T cells were prepared from CBA mice (The Jackson Laboratory, Bar Harbor, ME) with CD4 magnetic bead separation following the protocols provided by the supplier (Miltenyi Biotec, Auburn, CA).

Reagents

Viral IL-10 (vIL-10) were prepared as previously described (26). Human IL-10 (hIL-10), mIL-6, and IFN-γ were purchased from BD PharMingen (San Diego, CA). Biotin-labeled anti-mouse IFN-γ, IFN-γRβ, and PE-labeled anti-mouse IL-10R1 Ab were purchased from BD PharMingen. The dominant-negative STAT3 construct Y705F (29) was kindly provided by C. Horvath (Mount Sinai School of Medicine). Anti-phosphorylated STAT1, anti-phosphorylated STAT3, and anti-STAT1 and anti-STAT3 Abs were purchased from New England Biolabs (Beverly, MA).

Northern blot hybridization and RT-PCR analysis

Total RNA was isolated from Ba/F3 transfected with mIL-10R1, MC9 cell lines, and CD4+ T cells by an acid guanidium thiocyanate-phenol-chloroform extraction method, and poly(A)+ RNA was purified using RiboSep mRNA isolation kit (Genome Therapeutics, Waltham, MA). Northern blot hybridization was performed as recommended by protocol with the PerfectHyb Plus hybridization buffer system (Sigma-Aldrich, St. Louis, MO). Full-length SOCS1 and SOCS3 probes were generated from plasmids pEF-FLAG-I/m-SOCS1 and pEF-FLAG-I/mSOCS3, which were kindly provided by D. Hilton (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The full-length SOCS2 probe was generated by RT-PCR and the β-actin probe was purchased (Sigma-Aldrich). Each lane contained 10 μg of mRNA. For reverse transcription, total RNAs were isolated from various cell types by using TRizol reagent (Life Technologies) as per protocol, and cDNAs were synthesized using random primers. PCR was performed, using serial dilutions of cDNA to ensure equal amounts of cDNAs were used by comparing an internal standard of GAPDH. Gene-specific primers for PCR amplification are: mouse SOCS1, 5'-CGT CGA GCA GCC GAC AAT GCG ATC-3' and 5'-CGT AGT CCT CCA GCA GCT CAA-3' to generate a 450-bp product; mouse SOCS2, 5'-GGA AGT ATG ACT GTT AAT GAA GCC-3' and 5'-CCC AGA TCG TAC CGG TAC ATT-3' to generate a 388-bp product; mouse SOCS3, 5'-CGG CGT AGC ATG TGC ACC CAC AGC AAG-3' and 5'-TTT GGA TCC TTA AAG TGG AGC ATC ATA-3' to generate a 690-bp product; human SOCS1, 5'-GTA GCA CAC AAG CAC GTG GCA-3' and 5'-TGG AAG AGG CAG CAG TCG AAG CTC-3' to generate a 445-bp product; human SOCS3, 5'-ATG GTC ACC CAC ACC AGC TTT-3' and 5'-TCA CAC TGG ATG CGC AGG TTC-3' to generate a 293-bp product. The PCR were performed in a final volume of 50 μl containing 25 pmol of each primer, 2 μl of diluted cDNA, and 1.25 U of DNA polymerase (PerkinElmer, Wellesley, MA). The samples were amplified for 30 cycles at 94°C (1 min), 57°C (2 min), and 72°C (3 min). Five microliters of each sample was separated by 2% agarose gel electrophoresis, and DNA was visualized by ethidium bromide staining.

Transfection of SOCS, IL-10R1, and STAT3 (Y705F) constructs in cell lines

SOCS1, SOCS2, and SOCS3 genes were amplified by PCR and cloned to the pRc/Res-EF vector, which contains an internal ribosome entry site and permits both SOCS gene and the enhanced green fluorescence protein (EGFP) gene to be translated from a single bicistronic mRNA (Clontech, Palo Alto, CA). Electroporation was performed and green fluorescence protein-positive cells were then sorted by flow cytometry and expression of SOCS molecules was confirmed by Northern blotting. The C-terminal deletion IL-10R1 mutants Δ402–559 without two distal tyrosines (Y427 and Y477), or Δ433–559 without tyrosine 477, were generated by PCR and transfected into Ba/F3 as previously described (24, 26). Electroporation was used to transfect mouse IL-10R1 into M1 or U3A cells, and IL-10R1 plus dominant-negative STAT3 at a ratio of 1:10 into U3A cells. Positive cells were then sorted by flow cytometry.

Western blot analysis

Protein extracts were prepared from various groups with cell lysis buffer (24, 26) and concentrations were determined by BCA assay (Pierce, Rockford, IL). Proteins were then separated by 7.5% SDS-polyacrylamide and transferred to polyvinylidene difluoride membranes to probe with various anti-STAT Abs. For IL-10-mediated inhibition of IFN-γ signal, cells were treated with 100 ng/ml IL-10 for 1 h and then treated with various doses of IFN-γ for another 15 min, and then STAT1 phosphorylation was analyzed. For transfected cell lines, cells were treated with IL-10, IL-6, or IFN-γ as indicated at a range of concentrations for 15 min, and then STAT1 or STAT3 phosphorylation was determined.

Proliferative responses

Cells expressing SOCS1 or SOCS3 were rested in RPMI with 10% FCS medium overnight, and then 5 × 10⁴ cells were plated in 96-well flat-bottom plates (Corning, Corning, NY) in 200 μl. hIL-10 was added as indicated at a range of concentrations. Cells were incubated with stimuli for 24 h, and proliferation was assessed 24 h after adding 10 μl of Alamar blue (AccuMed International, Chicago, IL) to each well, and plates were read at OD530–650 as previously described (26).

Assays for M1 cell growth arrest and differentiation

Cells (1 × 10⁴) were cultured in flat-bottom 96-well plates, with or without the indicated amounts of IL-6, IL-10, or IFN-γ for 3 days, and cell viability was determined by trypan blue exclusion. Cells were also collected after 5 days of cytokine treatment, and following cytokine deprivation they were subjected to May-Grünwald-Giemsa staining. Morphological differentiation was determined by counting 500 cells on stained cytospin smears and scoring the proportion of macrophages. Results of all experiments represent the mean of at least three independent determinations.

Results

IL-10 induces both SOCS1 and SOCS3 expression

To elucidate the role of the SOCS molecules in IL-10 signaling, we first examined the expression of SOCS1, SOCS2, and SOCS3 mRNAs in a pro-B cell line after cytokine treatment. Ba/F3 cells transfected with mIL-10R1 were plated in serum- and growth factor-free medium for 4 h and then treated with hIL-10. As expected, hIL-10 is able to rapidly induce SOCS3 mRNA expression within 30 min. Expression peaks at 45 min and is maintained for at least 90 min (Fig. 1A). These results are consistent with the report of SOCS3 gene induction by IL-10 in human monocytes and PMN (21, 22). After stripping the SOCS3 probe, the membranes were then rehybridized with a SOCS1 specific probe. SOCS1 mRNA is also induced by hIL-10 with similar kinetics to SOCS3, rapidly appearing within 30 min, and maintaining expression for at least 90 min (Fig. 1A). Subsequent reprobing for SOCS2 shows that SOCS2 transcripts are constitutively and variably expressed with no consistent changes observed after IL-10 stimulation (Fig. 1A). vIL-10 is the EBV IL-10 homolog, with 1000-fold lower receptor affinity compared with cellular IL-10. It has previously been shown to have activities similar to those of cellular IL-10 on Ba/F3 cells, but distinct activities on MC9 cells as a result of differential cellular receptor densities and ligand affinities (24, 26). Northern
blotting was performed on mRNA from cells treated with vIL-10. As shown in Fig. 1B, vIL-10 induces both SOCS1 and SOCS3 transcription in Ba/F3 transfected mIL-10R1 cells, but fails to induce either SOCS1 or SOCS3 expression in MC/9 cells. Treatment of MC/9 cells expressing high levels of mIL-10R1 (24) with vIL-10 showed that increased cell surface receptor density renders MC/9 cells responsive to vIL-10, so that both SOCS1 and SOCS3 are induced by vIL-10 in a similar fashion. Additional controls also show that only SOCS1, but not SOCS3, is induced by treatment of Ba/F3 or MC/9 cells with IFN-γ, confirming the specificity of the SOCS1 and SOCS3 probes and gene induction (Fig. 1C). Because vIL-10 does not induce SOCS1 or SOCS3 expression in parental MC/9 cells, this also suggests that IL-10-induced SOCS1 expression is likely not due to IFN-γ or other cytokines contaminating the samples.

To investigate whether these findings could be extended to primary nontransformed cells, we used magnetic beads to isolate splenic CD4+ T cells with at least 95% purity and expressing IL-10R1 (not shown). Cells were treated with hIL-10, mIFN-γ, or mIL-6 for 1 h, and RT-PCR for SOCS was then performed. The results were normalized to GAPDH RT-PCR products using serial dilutions to ensure equivalent amounts of cDNA in each reaction. As shown in Fig. 1D, IL-10, IFN-γ, and IL-6 all induce SOCS1 expression, and the signal induced by IFN-γ is stronger than that of IL-10 or IL-6. SOCS2 is variably expressed, and no consistent changes in its expression were observed in response to any of the cytokine treatments. SOCS3 is strongly induced by IL-10 and IL-6, and is weakly induced by IFN-γ treatment. These results confirm that IL-10 is also able to induce SOCS1 expression in primary cells, in addition to SOCS3 expression.

The induction of SOCS by IL-10 is dependent on STAT3 activation

To understand the molecular basis of IL-10-induced SOCS1 and SOCS3 expression, we generated Ba/F3 cell lines expressing mutant IL-10R1. It has been previously demonstrated that two distal tyrosine residues (Y427 and Y477) in the cytoplasmic domain of the mIL-10R are redundant and either one is required for receptor function and for activation of STAT3, but not for STAT1 or STAT5 (25). By using Ba/F3 cells expressing the C-terminal deletion IL-10R1 mutants Δ402–559 without the two distal tyrosines (Y427 and Y477) or Δ433–559 without tyrosine 477, we determined whether IL-10-induced SOCS1 and/or SOCS3 requires STAT3 activation, or whether SOCS induction depends on other activated STATs. Stable cell lines were generated and flow cytometric staining was used to ensure comparable receptor expression (not shown). As shown in Fig. 2A, STAT3 phosphorylation was observed only in cells expressing full-length mIL-10R1 or IL-10R1Δ433–559, but not IL-10R1Δ402–559. STAT1 and STAT5 phosphorylation were observed in all groups, although the STAT1 and STAT5 activation observed in cells expressing IL-10R1Δ402–559 is weaker, suggesting that STAT1 and STAT5 activation may be partially dependent on STAT3 activation. Northern blotting performed on the cells expressing the different forms of IL-10R1 confirmed that both SOCS1 and SOCS3 were induced by IL-10 only in cells expressing full-length IL-10R1, or cells expressing at least one distal tyrosine (IL-10R1Δ433–559) (Fig. 2B). Thus, both SOCS1 and SOCS3 induction are dependent on STAT3 activation.

It has been previously demonstrated (24, 25, 26) that IL-10 activates STAT1, STAT3, and STAT5 in Ba/F3 cells, and STAT1 and STAT3, but not STAT5, in MC/9 cells. To define the role of STAT5 in SOCS induction, dose-response studies were performed in both Ba/F3 and MC/9 cells, and showed that IL-10 induces SOCS1 and SOCS3 expression in a similar fashion in both lines.
The induction signal is stronger in Ba/F3 cells, compared with that of MC/9 cells. SOCS1 is induced in Ba/F3 by IL-10 at 0.3 ng/ml, similar to SOCS3 induction, while 10- to 100-fold higher concentrations of IL-10 are required to produce an equivalent response in MC/9 (Fig. 2C). This is likely due to the fact that mIL-10R1-transfected Ba/F3 cells have a much higher cell surface density of IL-10R1 compared with the constitutive low-level expression by MC/9, and therefore respond to lower concentrations of ligand (24). The results further demonstrate that IL-10-induced SOCS expression is not dependent on STAT5 activation.

To define the role of STAT1 and STAT3 in IL-10-mediated SOCS induction, STAT1-deficient U3A cells were transfected with mIL-10R1 receptor alone or combined with a dominant-negative STAT3 construct (Y705F) at a ratio of 1:10. IL-10R1-positive cells were selected by flow cytometry (29). Cells were treated with 100 ng/ml hIL-10. Western blotting for STAT3 activation confirmed responsiveness of these cells to IL-10, and the dominant-negative STAT3 coexpression prevented STAT3 activation (Fig. 2D). RT-PCR revealed that SOCS1 and SOCS3 were up-regulated after cytokine treatment in cells that lack STAT1 activation, but not in cells expressing STAT3 (Y705F) without STAT3 activation (Fig. 2E). These results demonstrate that IL-10-induced SOCS expression requires STAT3 activation. Taken together, the findings suggest that STAT3, but not STAT1 or STAT5, activation is required for rapid SOCS1 and SOCS3 transcript induction by IL-10, consistent with previous findings in STAT3−/− macrophages that STAT3 is required for all IL-10 functions, including anti-inflammatory responses (30).

SOCS1 and SOCS3 inhibit IL-10-mediated STAT3 activation and proliferative responses

To address the role of SOCS molecules in IL-10-mediated signaling and biological responses, SOCS1, SOCS2, or SOCS3 genes were transfected to Ba/F3 cells. We used the pIRES2-EGFP vector to permit flow cytometric sorting for EGFP and to obtain cells expressing different SOCS molecules and to overcome the low transfection efficiency of SOCS. Northern blotting was used to confirm positive cell transfection (Fig. 3A).

The effect of SOCS1, SOCS2, or SOCS3 proteins on IL-10-induced activation and tyrosine phosphorylation of STAT3 is shown in Fig. 3B. The results demonstrate that in control Ba/F3 cells, hIL-10 strongly activates STAT3. Constitutive expression of SOCS1 or SOCS3, but not of SOCS2, is able to partially block IL-10-mediated tyrosine phosphorylation of STAT3. As a control, IFN-γ-induced STAT1 activation is also inhibited by both SOCS1 and SOCS3, but not by SOCS2, consistent with previous reports (20). This finding is congruent with previous reports that SOCS1 and SOCS3 are able to block IL-6 or Oncostatin M-induced

FIGURE 2. IL-10-mediated SOCS induction is STAT3 dependent. A, STAT phosphorylation by IL-10 in Ba/F3 cells expressing no, wild-type, or mutant mIL-10R1. Cells were treated with 100 ng/ml hIL-10 for 15 min, and STAT1, STAT3, and STAT5 phosphorylation were examined by Western blotting with specific Abs. B, Northern blotting of SOCS1 or SOCS3 in the same cells after IL-10 treatment. C, Dose response to hIL-10-induced SOCS1 or SOCS3 expression in MC/9 and Ba/F3 cells. Northern blotting 1 h after treatment with indicated doses. D, STAT3 activation by IL-10 in IL-10R1− or IL-10R1 plus dominant-negative STAT3 (Y705F)-transfected U3A cells. Cells were treated with 100 ng/ml cytokine for 15 min, and Western blotting was then performed for phosphorylated STAT3. E, RT-PCR analysis of SOCS molecules induced by IL-10. U3A mIL-10R1 with or without STAT3 (Y705F) cells were treated with 100 ng/ml the indicated cytokines for 1 h, and whole cellular RNA was amplified by RT-PCR for SOCS1 or SOCS3.
STAT3 activation in HeLa cells and M1 cells (20, 31). To determine whether SOCS1 or SOCS3 can also interfere with IL-10-mediated proliferative responses, these cells were treated with hIL-10 at various concentrations. Fig. 3C shows that SOCS1 or SOCS3 can partially suppress IL-10-mediated proliferative responses, suggesting that both SOCS1 and SOCS3 are involved in negatively autoregulating IL-10 biological responses.

IFN-γ pretreatment of M1 cells prevents IL-10-induced STAT3 activation and cell growth arrest

To further investigate whether SOCS1 negatively regulates IL-10 signaling and biological function, murine M1 leukemia cells were used. In these cells, IFN-γ induces high levels of SOCS1 induction, and is correlated with inhibition of STAT3 activation and
We first examined IL-10R expression and STAT3 activation in parental M1 cells, but were unable to detect either expression of IL-10R1 or STAT3 activation after IL-10 treatment (not shown). Using the approach outlined above, M1 cells were transfected with mIL-10R1, and stable cell lines were selected by flow cytometry. In these cells, IL-10, similar to IL-6, induced STAT3 activation, apoptosis, cell growth arrest, and differentiation (Fig. 4A and unpublished data). Consistent with previous reports (31) and the findings in other cell lines (Figs. 1 and 2), IFN-γ induced SOCS1 expression, while IL-10 and IL-6 induced SOCS1 and SOCS3 expression, and IFN-γ did not have any growth inhibitory effects on these cells (not shown). We then tested whether pretreatment with IFN-γ could have a negative regulatory effect on IL-10 signaling and biological function. M1 cells expressing mIL-10R1 were treated with 100 ng/ml IFN-γ for 12 h, and then stimulated with 2 ng/ml hIL-10 for various times. Western blotting for STAT3 phosphorylation showed that similar high levels of STAT3 activation were detected from 0.5 to 3 h, with or without IFN-γ pretreatment. In contrast, differences in STAT3 phosphorylation were observed after 3 h of IL-10 treatment, with activation of STAT3 decreasing rapidly in the IFN-γ-pretreated groups, while persistent STAT activation was observed in the control group without IFN-γ treatment. By counting the cell numbers 3 days after IL-10 stimulation, we also found that the IFN-γ-pretreated group has significantly more cells. Thus, pretreatment with IFN-γ rendered cells resistant to IL-10-mediated cell growth inhibition (Fig. 4C). These results further support the notion that persistent STAT3 activation plays a critical role in cell growth arrest and differentiation (31). Our data are consistent with previous findings of the regulatory role of SOCS1 in IL-6- and LIF-mediated differentiation and STAT3 activation (31), and suggest that physiological levels of SOCS1 are able to negatively regulate IL-10-mediated signal transduction and biological responses.

**IL-10-mediated inhibition of IFN-γ signaling**

Studies showed that IL-10 inhibits IFN-γ-induced STAT1 activation in human monocytes, and suggested the inhibition might be through SOCS3 induction (22). Because SOCS1 has been demonstrated to play a critical role in negatively regulating IFN-γ signal and function (17), the mechanism of inhibition by IL-10 of IFN-γ requires further investigation. Ba/F3 cells expressing mIL-10R1 were treated with 100 ng/ml hIL-10 for 60 min, and then treated with 1–100 ng/ml IFN-γ for 15 min. Western blotting was performed to detect IFN-γ-induced STAT1 phosphorylation. Consistent with findings in human monocytes, pretreatment of cells with IL-10 suppresses IFN-γ-stimulated STAT1 phosphorylation (Fig. 5A), indicating that this model system could be used to elucidate the mechanisms of IL-10-mediated inhibition on IFN-γ signaling.

We next examined the effect of IL-10 on IFN-γR expression to differentiate whether inhibition of STAT1 phosphorylation is due to down-regulating cell surface receptors. Flow cytometric analysis showed similar levels of IFN-γRα expression in cells treated with or without IL-10 (Fig. 5B). IFN-γRβ was also examined; its expression level was very low, but no differences were observed in cells treated with or without IL-10 (not shown). Similar observations were made in CD4+ T cells and MC/9 cells (not shown). These results indicate IL-10 does not acutely affect IFN-γ receptor complex expression. The inhibitory effects of IL-10 must be through downstream signaling elements.

If IL-10 exerts its inhibitory effect on IFN-γ signaling through induction of SOCS molecules, it is expected that this requires new protein synthesis. To determine this possibility, cells were treated with cycloheximide in combination with IL-10, before treatment with IFN-γ. Western blotting showed that cycloheximide completely prevents the ability of IL-10 to inhibit IFN-γ-induced STAT1 phosphorylation (Fig. 5C). Thus, the IL-10-mediated inhibitory effect on IFN-γ signaling requires de novo protein synthesis, likely through SOCS molecule synthesis.

The C-terminal 30-aa sequence of the intracellular domain of the IL-10R1 receptor contains at least one functionally critical serine required for inhibition of proinflammatory cytokine synthesis by IL-10 (30). To determine whether IL-10 negative regulation of IFN-γ signaling uses the same molecular mechanism, STAT1 phosphorylation by IFN-γ in cells expressing no mIL-10R1, IL-10R1Δ302–559, IL-10R1 IL-10R1Δ302–559, or full-length receptor was examined. Similar inhibition was observed in cells expressing receptor with or without C-terminal serine residues (Fig. 5D), demonstrating that the inhibitory effects of IL-10 on IFN-γ signaling are independent of those serine residues, despite prior evidence showing them to be critical for other IL-10-mediated
anti-inflammatory activities (30). Thus, IL-10-mediated inhibition of IFN-γ signaling and anti-inflammatory responses use distinct mechanisms. Inhibition of IFN-γ signaling could be directly through induction of SOCS, while additional molecules and/or pathways may be required for anti-inflammatory activity.

**Discussion**

In the current study, we show for the first time that SOCS1 molecules are up-regulated by IL-10, and the results are consistent in various cell lines and primary CD4+ T cells. The finding that vIL-10 is also able to induce SOCS1 or SOCS3 in Ba/F3 or MC/9 cells that display increased cell surface receptor density further demonstrates that the induction is IL-10 specific and not due to cytokine contamination. Numerous results have shown that the SOCS induced by a single ligand depend on the target cell type and different SOCS molecules can be induced by the same ligand (32). Previous reports showed that only SOCS3 mRNA could be induced by IL-10 in human monocytes and PMN (21, 22), while SOCS2, SOCS3, and cytokine-inducible SH2 protein mRNA could be induced in the liver (33). The apparent contradiction with current results could be due to species or cellular differences or Northern blotting techniques, because we used purified mRNA instead of total RNA. Our probes, primers, and assays were further controlled by results showing that IL-10 fails to induce SOCS2, that IFN-γ induces SOCS1 but not SOCS3, and that IL-6 induces SOCS1 and SOCS3.

SOCS1 was initially identified as an inhibitor of IL-6-induced differentiation of murine leukemia M1 cells (34), as a JAK-binding protein (32), and a STAT-induced STAT inhibitor-1 protein with an SH2 domain similar to that of STAT proteins (34). Stimulation by IL-6 or LIF of M1 cells induced SOCS1 mRNA expression, and was blocked by transfection of a dominant-negative mutant of STAT3, demonstrating that IL-6-induced SOCS1 expression is mediated by STAT3 (34). Similar to IL-6, IL-10 activates STAT1, STAT3, and, in some cells, STAT5, and it has been well defined that STAT3 is critical for IL-10 signaling and function (24, 25, 26, 30). SOCS gene induction is observed only in cells expressing IL-10R receptors that are able to induce STAT3 phosphorylation after IL-10 treatment; mutant IL-10R that activate STAT1 and STAT5, but not STAT3, do not induce either SOCS gene (Fig. 2B). Studies in U3A cells, which are deficient in STAT1, show that IL-10 is able to induce SOCS1 and SOCS3 independent of STAT1. A dominant-negative construct prevents STAT3 activation and induction of SOCS molecules in U3A cells, showing that up-regulation of SOCS by IL-10 is STAT3 dependent. Studies in MC/9 cells, in which IL-10 does not activate STAT5, demonstrate that IL-10-mediated SOCS gene expression is not dependent on STAT5 activation. Taken together, our results demonstrate the critical role of STAT3 activation in IL-10-regulated SOCS induction, and display a similarity between IL-10 and IL-6 signal transduction. Thus, it could be anticipated that IL-10 uses the same mechanism as IL-6 and induces SOCS1 through STAT3 activation.

SOCS1 binds to all four JAKs (JAK1, JAK2, JAK3, and tyrosine kinase 2) through its central SH2 domain and inhibits their kinase activity in vitro. When the SOCS1 protein is overexpressed in cell lines, it can inhibit STAT activation induced by several cytokines, including IFNs, IL-6, IL-4, and LIF (for review, see Ref. 32). SOCS3 has been implicated in the negative signaling of growth hormone, leptin, ciliary neurotrophic factor, IL-2, and IL-6 (18, 32), while SOCS1 has been implicated in the regulation of signaling by IFN-γ (17, 20, 32). We hypothesized that SOCS induction would also be an important negative regulatory mechanism induced by IL-10. We showed that overexpression of SOCS1 or SOCS3 in Ba/F3 cells is able to block not only IFN-γ signaling,

### FIGURE 5

Inhibition of IFN-γ-mediated STAT1 activation by IL-10 is not dependent on carboxyl-terminal serine residues. **A**, Pretreatment with IL-10 inhibits IFN-γ-induced STAT1 activation. Ba/F3 cells with or without IL-10 pretreatment for 60 min, followed by the indicated doses of IFN-γ for 15 min. Western blotting for total STAT1 and phosphorylated STAT1. **B**, Pretreatment with IL-10 does not cause IFN-γR down-regulation. Cells, with or without 100 ng/ml IL-10 treatment for 1 h, were stained for IFN-γRα by flow cytometry. **C**, Inhibitory activity of IL-10 toward STAT1 activation by IFN-γ requires new protein synthesis. Cells were incubated for 1 h with 100 ng/ml IL-10 alone or in combination with 10 μg/ml cycloheximide, and stimulated for 15 min with IFN-γ, and STAT1 phosphorylation was then examined by Western blotting. **D**, STAT1 phosphorylation by IFN-γ is inhibited in cells expressing mutant mIL-10R1 deleted in the carboxyl-terminal Δ433–529 and therefore lacking the serine residue. Cells expressing various truncated mIL-10R1 were treated with 100 ng/ml IL-10 for 1 h, and then treated with or without 1 ng/ml IFN-γ. Western blotting was performed as previously described.
but also IL-10-mediated STAT3 activation and proliferative responses, suggesting that both SOCS1 and SOCS3 are involved in negative autoregulation of IL-10 signaling and function. These data are consistent with previous reports that overexpression of SOCS1 or SOCS3 blocks IL-6- and Oncostatin M-induced STAT3 activation in HeLa cells (20), and forced overexpression of SOCS3 in J774 cells inhibits IL-10-mediated STAT3 activation (23).

To further determine whether SOCS1 induction alone could be sufficient to block IL-10-mediated signal transduction and biological activities, we studied the M1 cell line. After IL-10R1 transfection, we showed that IL-10 then is able to induce STAT3 activation, apoptosis, cell growth arrest, and differentiation in a fashion similar to that of IL-6 in M1 cells, and thus established a useful model to define the role of SOCS1 in IL-10-mediated signaling and biological functions. The results showed that, although pretreatment with IFN-γ failed to inhibit initial STAT3 activation, it clearly blocked persistent STAT3 activation at later time points, which has been shown to be critical in cell growth inhibition and differentiation. Therefore, these data suggest the involvement of SOCS1 in negative regulation of IL-10 function. We also studied primary mouse CD4 T cells, and pretreatment with IFN-γ also failed to inhibit IL-10-induced initial STAT3 activation (data not shown). Because STAT3 activation is not persistent in CD4 cells, we are not able to define the role of SOCS1 in negatively regulating IL-10 signaling and function in these cells. These data suggest that the mechanisms regulating IL-10 signals might depend on cell types. Additional signals or molecules might also be required to fully suppress IL-10 signaling. It has been shown that overexpressed protein tyrosine phosphatase eC inhibits IL-10- and IL-6-induced STAT3 activation (35), and inhibition of IL-6- or IL-10-induced STAT activation by inflammatory cytokines and stress factors is mediated by the p38 subfamily of stress-activated protein kinases (36–38). Therefore, autoregulation of IL-10 signaling is likely to be more complex than SOCS feedback inhibition.

Characteristic functions of IL-10 are that it suppresses proinflammatory cytokine (TNF-α, IL-1, IL-6, and IL-12) production, inhibits IFN-γ synthesis, and negatively regulates cell-mediated immune responses. Studies have shown that SOCS1 and SOCS3 have overlapping activities, and both are induced by and inhibit the actions of a similar spectrum of cytokines (32). However, the phenotype of SOCS1−/− mice demonstrates a key role for SOCS1 in regulating IFN-γ responses, which cannot be compensated by SOCS3 (17, 18). Thus, SOCS1 is the major inhibitor of IFN-γ signaling. IL-10 suppresses the ability of LPS to increase transcription of IL-1, TNF-α, IL-6, and other cytokines or cytokine receptors, and the ability of IFN-γ to enhance the effects of LPS can also be suppressed by IL-10. We report in this study that pretreatment of various cell lines with IL-10 increases the level of SOCS1 and directly inhibits IFN-γ signaling and STAT1 activation. Inhibition also requires novo protein synthesis. Because both SOCS1- and SOCS3-deficient phenotypes are embryonic or neonatal lethal, our results cannot formally differentiate whether inhibition by IL-10 of IFN-γ signaling is through SOCS1, SOCS3, or both. However, given the critical role of SOCS1 in regulating IFN-γ function, we conclude that IL-10-induced SOCS1 must play an important role in inhibition of IFN-γ activity.

Studies on macrophages derived from mice engineered to express a genetic STAT3 deficiency in the myeloid cell compartment establish that STAT3 activation is required for all IL-10-mediated responses, but its activation alone is not sufficient for IL-10 to mediate anti-inflammatory responses (30). The C-terminal 30-aa sequence of the intracellular domain of the receptor contains at least one functionally critical serine required for inhibition of proinflammatory cytokine synthesis by IL-10 (30). We show in this study that IL-10 is able to active STAT3 and induce SOCS1 and SOCS3 expression in cells expressing mutant IL-10R1 deleted in the carboxyl-terminal Δ433–529 and therefore lacking the serine residues. These results demonstrate that SOCS activation is independent of the serine residues, and suggest that SOCS activation alone is not sufficient for the anti-inflammatory functions of IL-10. Additional experiments showed that inhibition of IFN-γ-induced STAT activation is also not dependent on those serine residues. These results indicate IL-10-mediated inhibition of IFN-γ signaling and inhibition of proinflammatory cytokine synthesis use distinct mechanisms (35, 39). The result in this study that IL-10 is able to induce SOCS1 expression provides a direct link between IL-10 signaling and IFN-γ signaling. Generally considered as a Th2 cytokine, IL-10 is able to affect Th1/Th2 balance, promote Th2 development, and inhibit Th1 differentiation. It has been shown that inhibition of Th1 differentiation by IL-6 is mediated by SOCS1 (40). It is possible that SOCS1 also plays an important role in regulating Th1/Th2 immune derivation by IL-10, directly inhibiting IFN-γ signals and Th1 differentiation. Taken together, our findings provide new insights into understanding IL-10 signaling, Th cell differentiation, and anti-inflammatory activities.

Acknowledgments

We thank Drs. D. Hilton and T. Willson for kindly providing the mouse SOCSs and SOCS3 cDNA, Dr. G. Stark for generously providing the U3A cells, and Dr. C. M. Horvath for providing the dominant-negative STAT3 construct. We also thank Dan Chen and Miwei Mao for their excellent technical assistance.

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The Journal of Immunology


