Suppressor of Cytokine Signaling 3 Regulates Proliferation and Activation of T-helper Cells

Suppressors of cytokine signaling (SOCS) have been implicated in regulation of T-cell activation and cytokine-mediated differentiation of T-helper cells. In this study we have characterized the pattern of SOCS expression in naive and activated primary T-helper cells, examined whether expression of SOCS genes is regulated by cytokine or T-cell receptor signaling, and analyzed the function of SOCS in differentiated T-cells. We show that SOCS1, SOCS2, SOCS3, CIS (cytokine-induced SH2 protein) genes are constitutively expressed in naive T-helper cells, with SOCS3 being the most abundant. Antigen stimulation of naive T-helper cells down-regulates SOCS3 expression and concomitantly up-regulates SOCS1, SOCS2, and CIS gene transcription, suggesting that SOCS genes are regulated differentially by T-cell activation. Down-regulation of SOCS3 expression is subsequently followed by gradual increase in SOCS3 level and corresponding decline in interleukin 2 (IL-2) secretion. In fact, SOCS3 mRNA levels are inversely correlated with the amount of IL-2 secretion and proliferative responses of differentiating T-helper cells, suggesting mutually antagonistic effects of SOCS3 and IL-2 and feedback regulation of T-cell activation by SOCS3. Furthermore, the degree of SOCS3 inhibition is antigen concentration-dependent and is mediated in part by growth factor independence-1, a T-cell transcription factor that regulates S-phase entry in T-cells. Forced overexpression of SOCS3 inhibits proliferation of T-helper cells, whereas depletion of endogenous SOCS3 by antisense SOCS3 cDNA enhances T-cell receptor- and cytokine-induced proliferation. Taken together, these results suggest a role for SOCS3 in maintaining T-helper cells in a quiescent state. Transient inhibition of SOCS3 by antigen stimulation may therefore be essential in allowing activation of resting T-cells.

When a naive T-helper (Th) cell encounters its cognate Ag for the first time, it undergoes differentiation into a Th1 or a Th2 effector cell type. Th1 cells predominantly secrete interferon γ (IFNγ) and lymphotoxin (tumor necrosis factor β) and induce delayed type hypersensitivity responses whereas Th2 cells produce mainly IL-4, IL-5, and IL-10 and promote humoral responses (1, 2). Uncontrolled activation of naive T-helper cells can lead to exaggerated Th1 or Th2 responses and may predispose the host to autoimmune or allergic diseases, respectively (1, 2). Thus, understanding factors that regulate naive T-helper cell activation has implications for development of more effective vaccines, as well as immune modulation therapies against allograft rejection and autoimmune and allergic diseases (2). Although factors such as the dose of the stimulating Ag and nature of the antigen presenting cell (APC) clearly play important roles in inducing the quiescent naive T-helper cells to enter the G1 phase of the cell cycle, progression through the cycle in response to cytokines and growth factors such as IL-2 is under stringent regulation. Mechanisms involved in this regulation include negative regulatory receptors (CTLA-4 and FAS), cytoplasmic inhibitory proteins (Csk, SHP-1, and SHIP-2) (3–5), and suppressors of cytokine signaling (SOCS) (6, 7). SOCS proteins are of particular interest because of their critical roles in regulating cytokine and growth factor signaling (6, 7). Recent studies showing that SOCS proteins interact with proteins that play critical roles in T-cell activation (e.g. ZAP70, Gfi-1, and calcineurin) suggest that they may play a role in the regulation of essential aspects of the T-helper cell activation program.

SOCS are a newly described family of intracellular cytokine-inducible negative feedback regulators that target cytokine receptors and cytoplasmic signaling adaptor molecules (6, 7). The family is comprised of eight members characterized by the presence of an Src homology 2 domain and a carboxyl-terminal conserved domain called the SOCS box (8–10). In addition to functioning in a classical feed back regulatory loop, SOCS proteins can also inhibit responses to cytokines that are different from those that induce their expression. Their inhibitory effects derive from direct interaction of the SOCS proteins with cytokine receptors and/or Janus kinases, thereby preventing recruitment of signal transducers and activators of transcription to the signaling complex (11–14). SOCS proteins also regulate protein turnover by targeting proteins for polyubiquitination and proteosome-mediated degradation (8). SOCS1−/− mice have defective thymocyte development (15, 16), and SOCS3 is highly expressed in Th2 cells (17, 18), underscoring the involvement of SOCS proteins in T-cell development.

In a previous report (17) we showed that differentiation of naive T-helper into mature Ag-specific Th1 or Th2 phenotype is associated with preferential expression of SOCS1 and SOCS3, respectively. In this study, we sought to determine whether transcription of SOCS genes is regulated by TCR in primary responses of naive T-helper cells. We show that SOCS genes

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‡ The abbreviations used are: Th, T-helper; Ag, antigen; HEL, hen egg lysozyme; SOCS, suppressor of cytokine signaling; CIS, cytokine-induced Src homology 2 protein; Gfi-1, growth factor independence-1; IFN, interferon γ; IL, interleukin; Ab, antigen present inducing cell; Ab, antibody; RT, reverse transcriptase; TK, thymidine kinase; TCR, T-cell receptor.
are constitutively expressed in naive cells and differentially regulated by T-cell activation. An early event in the activation of naive T-helper cell is a transient down-regulation of SOCS3, and the degree of SOCS3 inhibition is inversely correlated with the levels of T-cell proliferation and IL-2 secretion. We further show that the transient down-regulation of SOCS3 may be mediated in part by increased expression of Gr-1, a positive regulator of T-cell proliferation, and a suppressor of SOCS genes transcription (19, 20). Overexpression of SOCS3 in Th2 cells inhibits T-cell proliferation while endogenous SOCS3 enhances proliferation. These results suggest that SOCS3 may function in concert with other proteins to maintain quiescent state in lymphocytes, and transient inhibition of SOCS3 expression may therefore be essential to allow resting T-cells to respond to growth signals and proliferate.

EXPERIMENTAL PROCEDURES

Isolation and Functional Characterization of Naive and Activated T-helper Cells—CD4+ T-cells were isolated from spleens and lymph nodes of hen egg lysozyme (HEL)-specific TCR transgenic mice (a gift from Dr. Mark Davis, Stanford University) by a combination of T-cell enrichment columns and magnetic sorting as described previously (17). By fluorescence-activated cell sorter analysis, the cells were found to express high levels of CD62L (>95) and low levels of CD25 (<6%), features associated with naive T-helper cell phenotype. The cells were cultured in RPMI 1640 medium supplemented with 50 μM 2-mercaptoethanol, antibiotics, and 10% fetal bovine serum and stimulated with 0.1, 1.0, or 10 μg/ml HEL (Sigma) in the presence of 10 ng/mL irradiated AttaC, Manassas, VA, was cultured in RPMI 1640 medium supplemented with 2 mM t-glutathione, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 μg/ml IL-10 (R & D Systems), 10% rat T-STIM factor with concanavalin A (BD Biosciences) as recommended. Cells (2 × 10^5 cells/ml) were stimulated in anti-CD3 Ab-coated T-cell activation plate (BD Biosciences) plus anti-CD28 Ab (5 μg/ml).

Cytokine Measurements— Supernatants collected after 48 or 96 h of stimulation of naive T-helper cells with HEL and APC were assayed for cytokine secretion by enzyme-linked immunosorbent assay, using kits from R&D Systems (Minneapolis, MN). To determine the extent to which HEL-specific T-cell proliferation was mediated by TH1 vs. TH2 cytokines, the proliferation of HEL-stimulated T-helper cells was compared with that of cells cultured in medium containing 1% rat T-STIM. After 2 or 4 d, the cultures were pulsed with 3H-thymidine (0.5 Ci/mM) for 16 h and harvested on filter plates. Triplicate cultures were subjected to a final 10-min extension at 72°C.

Proliferation Assay—Lymphocyte proliferation assay was performed as detailed elsewhere (21). Briefly, freshly isolated CD4+ or CD8+ T cells or Th2 D10.G4.1 line cells (2.5 × 10^5 cells/well) were stimulated with HEL (0.1, 1.0, or 10 μg/ml) or anti-CD3/CD28 Abs (Pharmin- gen). The D10.G4.1 Th2 cell line (ATCC, Manassas, VA) was cultured in RPMI 1640 medium supplemented with 2 mM t-glutathione, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 μg/ml IL-10 (R & D Systems), 10% rat T-STIM factor with concanavalin A (BD Biosciences) as recommended. Cells (2 × 10^5 cells/ml) were stimulated in anti-CD3 Ab-coated T-cell activation plate (BD Biosciences) plus anti-CD28 Ab (5 μg/ml).

Quantitative RT-PCR Analysis—First strand cDNA synthesis was performed as described previously (17). A negative control reaction without reverse transcriptase was performed for each RNA sample, and RNA samples were normalized to 18 S RNA using Taqman ribosomal RNA control reagents kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI 7700 (Applied Biosystems) or iCycler iQ real time PCR sequence detection system (Bio-Rad, Hercules, CA) as described previously with the following forward and reverse primers, respectively: for SOCS1, 5'-ATGTTGGACGTTGAGAACCGG-GAAACAGG-3' and 5'-CCTCTTTGGTTCCAGCTAC-3' for SOCS2, 5'-AAGCCAATCTTCCAGTGTAAC-3' and 5'-GGTGTGCGCGAGGAAAGTG-3' for SOCS3, 5'-CCCTCTTGCTGACCAAACTGAG-3' and 5'-GCTCTCTCTCAGCTGCG-3' for CIS, 5'-CCCGAGCATCGACCCCTTCA-3' and 5'-CGTCTGGCCTATGACAGCAG-3' for CD4, 5'-GTTGAGGCTGAGAACTCTGTTCGTA-3' and 5'-ATGAACGATTGCGGCTTGAACAGC-3', and 5'-ACGCTGAGACACATTCTC-3' for CD4, 5'-GTTGAGGCTGAGAACTCTGTTCGTA-3' and 5'-CCTCTTTGGTTCCAGCTAC-3'. Amplification was carried out for 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension at 72°C for 30 s. After the 30th cycle the samples were subjected to a final 10-min extension at 72°C. First strand cDNA synthesis containing each mRNA sample but no reverse transcriptase was performed to control for possible DNA contamination with cDNA samples as targets for PCR amplification. PCR amplified fragments were fractionated on 0.1 or 1.5% agarose gels and stained with ethidium bromide. For SOCS3 and CD4, 10 μg digoxigenin-11-2'-deoxy-uridine-5-triphosphate (Roche Applied Science) was added to the PCR reaction, and the fractionated agarose gels were transferred onto nylon membranes. Amplification products were detected using anti-digoxigenin-AP conjugated chemiluminescence substrate, CDP-Star (Tropix Inc., Bedford, MA).

Western Blotting Analyses—Preparation of whole cell lysates and immunodetection were performed as described (17). Briefly, samples (40 μg/lane) were fractionated on 4–20% gradient SDS-PAGE and anti-SOCS3 or CD4, Gr-1, or anti-CD28 Abs (Pharmin-gen) were used as probes. Preimmune serum was used in parallel as controls, and signals were detected with horseradish peroxidase-conjugated secondary antibody (Pib9_Ab (Zymed Laboratories Inc.) using an ECL system (Amersham Biosciences).
Transient Transfection and Reporter Assays—Plasmids used for transfection are as follows: pGL3-basic (empty vector) reporter plasmid (Promega, Madison, WI), mouse SOCS3 promoter sequences ligated to the firefly luciferase reporter plasmid (pGL3-basic-SOCS3), Gfi-1 cDNA expression vector (pcDNA3.1-Gfi-1), and pRL-TK (Promega), which encodes Renilla luciferase. pGL3-SOCS3 promoter was kindly provided by Dr. Melmed, (Department of Medicine, UCLA) (23), and pcDNA-Gfi-1 is the full-length mouse Gfi-1 cDNA cloned into the BamHI and EcoRI sites of mammalian expression vector, pcDNA 3.1(H11001) (Invitrogen). The D10.G4.1 Th2 cells (0.5 × 10^6/0.5 ml) were co-transfected with pGL3-SOCS3 (1 μg/ml), pcDNA3.1-Gfi-1 (1 μg/ml), and pRL-TK (0.2 μg/ml) using LipofectAMINE 2000 according to the manufacturer’s instructions (Invitrogen). After 48 h of transfection, firefly and Renilla luciferase activities of the cell lysates were analyzed using the Dual-Luciferase® reporter assay system (Promega) with the Lmax luminometer (Molecular Devices, Sunnyvale, CA). The lysates were also evaluated for Gfi-1 gene expression by Western blot analysis. Renilla luciferase activities were used for internal sample normalization, and the indicated relative reporter activities take into account normalized transfection efficiency.

RESULTS
SOCS Genes Are Regulated Differentially by T-helper Cell Activation—We examined effects of TCR signaling on regulation of SOCS gene expression in naïve cells (Day 0), T-helper cells stimulated with Ag/APC for 2 (Day 2) or 4 (Day 4) days by real-time PCR. SOCS mRNA levels are presented as copies/cell as calculated in the text. B, SOCS expression as a ratio of SOCS copy number in Day 2 or Day 4 Ag-primed cells relative to the number in naïve T-helper cells. C, Northern blot analysis using 32P-labeled SOCS1 or SOCS3 cDNA as probe and RNA (20 μg/lane) from naïve or Day 2 Ag-primed T-helper cells. D, RT-PCR analysis of SOCS3 expression in naïve cells and T-helper cells stimulated with Ag/APC for 2 (Day 2) or 4 (Day 4) days. PCR products were detected by using anti-digoxigenin-AP conjugate and chemiluminescence substrate, CDP-Star. E, real-time RT-PCR analysis of SOCS3 mRNA level in T-helper cells cultured under non-polarizing (Tho) or neutral (Thn) condition as described under “Experimental Procedures.”

Fig. 1. Transcription of SOCS genes in T-helper cells is differentially regulated by Ag stimulation. A, quantitative detection of SOCS mRNAs in naïve cells (Day 0), T-helper cells stimulated with Ag/APC for 2 (Day 2) or 4 (Day 4) days by real-time PCR. SOCS mRNA levels are presented as copies/cell as calculated in the text. B, SOCS expression as a ratio of SOCS copy number in Day 2 or Day 4 Ag-primed cells relative to the number in naïve T-helper cells. C, Northern blot analysis using 32P-labeled SOCS1 or SOCS3 cDNA as probe and RNA (20 μg/lane) from naïve or Day 2 Ag-primed T-helper cells. D, RT-PCR analysis of SOCS3 expression in naïve cells and T-helper cells stimulated with Ag/APC for 2 (Day 2) or 4 (Day 4) days. PCR products were detected by using anti-digoxigenin-AP conjugate and chemiluminescence substrate, CDP-Star. E, real-time RT-PCR analysis of SOCS3 mRNA level in T-helper cells cultured under non-polarizing (Tho) or neutral (Thn) condition as described under “Experimental Procedures.”

Regulation of T-cell Activation and Proliferation by SOCS3

SOCS Genes Are Regulated Differentially by T-helper Cells Activated by HEL TCR transgenic (3A9) Mice. We show here by real-time quantitative 5′-nucleotide fluorogenic RT-PCR analysis that SOCS1, SOCS2, SOCS3, and CIS mRNAs are constitutively detected in naïve T-helper cells (Table 1, see Day 0), with SOCS3 being the most abundant SOCS member detected (Fig. 1A). Stimulation of the cells for 48 h with HEL (1.0 μg/ml) and APC induces a precipitous drop in SOCS3 mRNA level, whereas substantial increases are observed in the levels of SOCS1, SOCS2, and CIS mRNA transcripts (Fig. 1A). Analysis of cytokine secretion by these cells reveals a dramatic increase in IL-2 secretion, with minimal or no secretion of IFNγ, IL-4, IL-5, and IL-10 (data not shown) at this time point. By day 4 of antigenic stimulation, we begin to observe a steady increase of SOCS3 expression (Fig. 1), and this coincides with a sharp decline in IL-2 (data not shown). The levels of other SOCS members either remain essentially the same (SOCS1 and SOCS2) or experience a modest reduction (CIS) (Fig. 1A), and these temporal changes in the pattern of SOCS expression are accompanied by increases in the levels of secreted IFNγ, IL-5, and IL-10 (data not shown). Comparison of the levels of SOCS
expression in naïve cells to those of activated T-helper cells reveals substantial increases in SOCS1 and SOCS2 expression, as well as a modest increase in CIS level (Fig. 1B). In contrast, there is a net diminution in SOCS3 mRNA level (Fig. 1B).

Similar results were obtained from four independent Ag-primed naïve T-helper cell experiments. Because of the very high sensitivity of real-time RT-PCR assay, the observed inhibition of SOCS3 and changes in the pattern of SOCS genes expression may merely reflect events occurring in very few cells in our cultures. We therefore verified these results by a less sensitive technique that detects biologically relevant changes in gene expression. As indicated on the Northern blot autoradiogram (Fig. 1C), we detect SOCS3 expression in naïve T-helper cells but not in the cells stimulated with Ag for 48 h. On the other hand, we do not detect significant SOCS1 expression in naïve T-helper cells by this method, but do so after 48 h stimulation (Fig. 1C). Down-regulation of SOCS3 expression following Ag stimulation has been observed in three separate Northern blot experiments and conventional RT-PCR assays (Fig. 1D), clearly recapitulating our real-time quantitative RT-PCR and data.

To examine whether the transient inhibition of SOCS3 is mediated by cytokine signaling pathways activated by polarizing cytokines that are secreted by differentiating T-helper cells and APCs, we stimulated naïve cells for 48 h under non-polarizing (Tho) or neutral (Thn) condition. Regardless of whether exogenous neutralizing antibodies are absent (Tho) or present in the culture (Thn; anti-IL-4, anti-IL-12, anti-IFNγ Abs) we observe significant reduction in SOCS3 level following antigenic stimulation, suggesting that neither IL-4, IL-12, nor IFNγ is directly involved in the transient repression of SOCS3 expression (Fig. 1E). Collectively, these results indicate that SOCS genes are differentially regulated in Ag-primed T-helper cells. In contrast to other SOCS members, SOCS3 mRNA is transiently down-regulated in response to T-cell activation, and the repression of SOCS3 expression is not mediated by Th1 or Th2 polarizing cytokines.

The Degree of SOCS3 Repression Is Ag Dose-dependent and Correlates with T-helper Cell Proliferative Response—To determine whether the down-regulation of SOCS3 expression is induced by TCR-mediated signals, we stimulated naïve cells with various concentrations of the HEL in the presence of APC and analyzed their effects on SOCS3, as well as SOCS1 expression, by real-time RT-PCR. As shown on Fig. 2A, there is a substantial increase in the SOCS1 mRNA level following antigenic stimulation with each of the HEL concentrations used (Fig. 2A). In contrast to SOCS1, we observe a dramatic decrease in SOCS3 mRNA levels of cells stimulated for 2 days, and diminution in the SOCS3 mRNA levels is highly dependent on the Ag dose, being most striking at the higher Ag concentrations (Fig. 2A). However, in cells stimulated for 4 days we observe an increase in SOCS3 transcripts, suggesting that the inhibition of SOCS3 expression is transient and dependent on both the strength and duration of TCR signaling. These results have also been verified by three independent Northern blot analyses in which stimulation of naïve T-helper cells at the
higher concentrations of HEL (1.0 or 10.0 μg/ml) induced transient inhibition of SOCS3 (Fig. 2B). We further show that SOCS1 and SOCS3 proteins are constitutively expressed in naive Th cells (Fig. 2C, lane 1) and that antigenic stimulation induces a transient inhibition of SOCS3 but not SOCS1 (Fig. 2C, lane 3). Thus, in concert with our Northern and RT-PCR results (see Fig. 1 and Fig. 2, A and B), the antigen dose-dependent alterations in SOCS mRNA levels are also reflected in protein levels (Fig. 2C). Detection of transient repression of SOCS3 expression by the rather insensitive Northern blot assay and by Western blotting suggests that alterations in SOCS3 mRNA levels is a functionally relevant event during naive T-helper cell stimulation.

To study the role of alterations in SOCS3 mRNA levels in T-helper cell activation, we examined whether changes in the level of IL-2 production or in T-cell proliferative response correlate temporally with the degree of SOCS3 repression following stimulation of naive Th cells with 0.1, 1.0, or 10.0 μg/ml HEL in the presence of APC. At each time point examined we observe a positive correlation between the Ag dose and proliferative response (Fig. 2D) or ability to produce IL-2 (Fig. 2E). Interestingly, marked increases in proliferative responses and IL-2 production occur within the narrow window (day 2) that coincides temporally with the transient inhibition of SOCS3 expression (see Fig. 1 and Fig. 2, A and B). By day 4 and thereafter, when SOCS3 and other SOCS members are maintained at relatively high levels, the cells have equilibrated to lower proliferative rates. Moreover, we observe very little or no change in the proliferative response of cells stimulated with the HEL concentration (0.1 μg/ml) that induced minimal inhibition of SOCS3 expression. In fact, a net increase of ~44% in cellular proliferation is noted between day 2 and day 4 in the cells stimulated with 0.1 μg/ml HEL (Fig. 2D). In contrast, HEL concentrations that significantly inhibit SOCS3 expression on day 2 (1.0 and 10.0 μg/ml) have the highest growth rate at this time point and experience >40% decrease in growth by day 4 (Fig. 2D). The increase in the steady-state levels of SOCS3 and other SOCS members after 96 h of Ag stimulation (see Figs. 2 and 4) also coincides with diminished abilities to produce IL-2. By day 6 of Ag stimulation virtually all of the IL-2 are consumed by proliferating cells, leaving very little IL-2 in the supernatant (Fig. 2E).

SOCS3 Regulates TCR- and Cytokine-induced Proliferation of Differentiated T-helper Cells—Because the primary T-cells analyzed in this study have not fully committed to a Th1 or Th2 cell type, it remains a possibility that inhibition of SOCS3 by TCR signaling is functionally relevant to early differentiating T-helper cells but not mature differentiated Th1 or Th2 cells. The D10.G4.1 Th2 cell lines were therefore stimulated with anti-CD3/anti CD28 Abs and, as shown by Northern blot analysis, SOCS3 expression is down-regulated by TCR activation (Fig. 3A). Analysis of the AE7 Th1 cell lines produced similar results (data not shown), suggesting that inhibition of SOCS3 expression is coupled to T-cell activation of naive and resting T-helper cells. To determine whether SOCS3 directly regulates proliferation of T-helper cells, we generated D10.G4.1 Th2 cells with stable overexpression of sense or antisense SOCS3 cDNA and analyzed effects of these constructs on TCR- and IL-2-induced proliferation. The D10.G4.1 Th2 cells secrete IL-4, IL-5, and IL-10 but not IFNγ or IL-2 (data not shown) and are highly dependent on exogenous IL-2 for growth, consistent with previous reports (24, 25). The expression pattern of SOCS3 protein by representative clones is depicted in Fig. 3B. As indicated for the control clone (empty vector), proliferation of these cells, as assessed by 3H-thymidine incorporation, declines significantly as the amount of IL-2 in the culture is progressively consumed (Fig. 3C, day 2). TCR ligation and co-stimulation by anti-CD3/anti CD28 Abs leads to ~50% increase in the proliferative response, but the TCR contribution is lost as IL-2 levels decline (Fig. 3C, day 2). It should, however, be noted that detection of the exact amount of Ag-induced increase in proliferation is influenced by the amount of T-STIM factor in the medium. At high T-STIM concentration (10%) the background proliferation resulting from cytokine-mediated proliferation is relatively high and minimizes differences in TCR-induced proliferation between Ag-stimulated and unstimulated cells (data not shown). Nonetheless, in contrast to control cells, overexpressing SOCS3 blocks anti-CD3/CD28-mediated proliferation, suggesting that the inhibitory effects of SOCS3 targets TCR signaling pathways. Remarkably, depletion of SOCS3 levels by antisense cDNA enhances cellular proliferation; particularly the TCR-mediated response and the decrease in mitosis that result from consumption of IL-2 in the culture does not occur (Fig. 3C, day 2). The growth regulatory effects of SOCS3 are most evident in cells maintained in culture after exogenous IL-2 is consumed (Fig. 3C, day 4). Under this condition, control or SOCS3 clones stop dividing whereas the Th2 cells expressing the SOCS3(AS) construct maintain significantly higher level of proliferation, further underscoring the growth regulatory effects of SOCS3 on T-helper cells (Fig. 3C, day 4). To examine the possibility that the observed inhibition of Ag-induced proliferation by SOCS3 may be because of secondary effects of the other SOCS members, we analyzed the effects of the antisense and sense SOCS3 constructs on the other SOCS family members. As shown in Fig. 3D there is no significant difference in the level of SOCS1, SOCS2, or CIS between the control and SOCS3-transfected cells indicating that inhibition of Ag-induced proliferation derives from overexpression of SOCS3 and not because of secondary effects of the other SOCS members. On the other hand, increase in SOCS1 and CIS in the clones transfected with the antisense SOCS3 construct may reflect the fact that in this culture system, the cells produce IL-4, IL-5, and IL-10. Thus, in the absence of SOCS3 there appears to be a compensatory increase in the other SOCS members to effect the requisite feedback regulation of these cytokines. Together, these results, which have been verified in three independent experiments using different clones, provide compelling evidence for a role of SOCS3 in regulating TCR- and cytokine-mediated T-helper cell proliferation.

SOCS3 Inhibition Correlates with Down-regulation of p27kip1 and Up-regulation of Gfi-1—TCR ligation leads to activation of Src family kinases, followed by activation of ZAP70 and other downstream effectors that eventually trigger the MAPK cascade and activation of proteins that regulate gene transcription and cell cycle progression (26). One of these proteins is Gfi-1, a transcriptional repressor that accelerates S-phase entry of primary T-cells in response to antigenic stimulation (19). The SOCS3 gene contains multiple Gfi-1 sites in its promoter, and it has been shown to inhibit the transcription of SOCS genes (20). We therefore hypothesized that the down-regulation of SOCS3 may derive from up-regulation of Gfi-1 gene during T-cell activation. RNAs isolated from primary T-helper cells stimulated with various concentration of HEL were therefore analyzed to see whether there is a correlation between the levels of SOCS3 and Gfi-1 expression. As shown in Fig. 4, A and B, naive and T-helper cells stimulated with low concentration of HEL (0.1 μg/ml) for 2 days express lower amounts of Gfi-1. The cells stimulated at the higher concentrations express much higher levels, suggesting that Gfi-1 may contribute to the repression of SOCS3 gene transcription at the early time point of T-cell activation. Another protein that reg-
ulates cell cycle progression of T-cells is the cyclin-dependent kinase inhibitor p27\(^{kip1}\) (27). In contrast to Gfi-1, p27\(^{kip1}\) is down-regulated in cells stimulated for 2 days and to a lesser extent at the day 4 time point (Fig. 4A). To establish that the correlation between SOCS3 and Gfi-1 levels is also reflected at the protein level, Western blot analysis was performed on whole cell extracts isolated from Th cells stimulated with various concentrations of HEL. As shown in Fig. 2B, naive and T-helper cells stimulated with low concentration of HEL (0.1 μg/ml) for 2 days express lower amounts of Gfi-1 whereas cells stimulated at the higher concentrations express much higher levels. Together with the protein data shown in Fig. 2C, these data clearly recapitulate our RNA data. To examined whether the Ag-induced transient inhibition SOCS3 is mediated in part through Gfi-1, we transfected D10.G4.1 Th2 cells with a SOCS3 promoter construct and Gfi-1 cDNA expression vector. As shown in Fig. 4C, Gfi-1 significantly inhibits SOCS3 promoter activity, providing suggestive evidence that the up-regulated expression of Gfi-1 following antigenic stimulation of naive Th cells may contribute to down-regulation of SOCS protein levels in activated T-cells.

**Discussion**

In this study we have characterized the pattern of SOCS expression in naïve and activated primary T-helper cells and have examined the function of SOCS3 in differentiated T-helper cells. We show that SOCS genes are constitutively expressed in naïve cells and that activation of primary T-helper cells by Ag/APC induces a precipitous decline in SOCS3 expression and concomitant up-regulation of SOCS1, SOCS2, and CIS mRNA levels. The inhibition of SOCS3 expression is Ag concentration-dependent, as Ag concentrations that induce TCR signals of low signaling strength are unable to repress SOCS3 transcription. Inhibition of SOCS3 also occurs in primary T-helper cells stimulated in medium containing anti-IFNγ, anti-IL-4, and anti-IL-12 Abs, indicating that repression of this SOCS member is not mediated by polarizing cytokines induced during T-cell activation. These results suggest that transcript-
tional regulation of SOCS3 gene is coupled to T-helper cell activation and mediated primarily through signals induced by TCR ligation and co-stimulation. While this manuscript was in preparation, a paper was published (28) showing that stimulation of naïve T-helper cells by anti-CD3 Ab up-regulates SOCS3 expression, an observation that is opposite our results. However, in three separate experiments with freshly isolated primary T-helper cells, we consistently observed transient down-regulation of SOCS3 following Ag stimulation (Fig. 1). One possible explanation for the difference between these results might be that they used anti-CD3 to induce T-cell activation whereas in our study HEL TCR transgenic T-cells were activated with the cognate HEL Ag in the context of syngeneic MHC class II. We directly assess the effect of anti-CD3 stimulation on SOCS3 expression in three Th cell clones from the AE7 Th cell line and the parent AE7 line; regardless of whether the cells are stimulated with anti-CD3 or anti-CD3/anti-CD28 Abs, antigenic stimulation induced down-regulation of SOCS3 expression (data not shown). Taken together with results from a recent report by Matsumoto et al. (29) showing that stimulation of primary CD4⁺ T-cells with Ag (ovalbumin) or ovalbumin plus anti-CD28 inhibits SOCS3, preponderance of the evidence indicates that a transient inhibition of SOCS3 is induced by Ag stimulation.

An issue of particular interest relates to the biological relevance of changes in SOCS3 expression induced by antigenic stimulation. Comparison of the proliferative response of primary T-helper cells reveals that increases in cell proliferation coincides temporally with inhibition of SOCS3 expression, and the proliferation potential correlates with the degree of SOCS3 repression (Fig. 2). As T-cell proliferation is initiated by TCR-mediated signals and cytokine-mediated signaling, and both

### Table I

**Inverse relationship between SOCS3 expression level and IL-2 secretion/cell proliferation**

| HEL (μg/ml) | Naïve Th cell | Stimulated Th Cell (48 h) | Stimulated Th Cell (96 h) |
|------------|---------------|--------------------------|--------------------------|
| 0          | ±±±           | +                        | +                        |
| 0.1        | ±±±           | +                        | +                        |
| 1.0        | ±±±           | +                        | +                        |
| 10         | ±±±           | +                        | +                        |

Naïve T-helper cells were stimulated with HEL and APC.

![Figure 4](image-url)  
**Transcriptional regulation of Gfi-1 or p27kip1 genes by TCR signaling correlates temporally with down-regulation of SOCS3 expression.** A, RT-PCR analysis of Gfi-1, p27kip1, or β-actin gene expression in naïve cells (day 0) or primary T-helper cells stimulated with Ag (0.1, 1.0, or 10.0 μg/ml HEL)/APC for 2 (Day 2) or 4 (Day 4) days. B, detection of Gfi-1 or β-actin protein expression in primary T-helper cells stimulated with Ag (0.1, 1.0, or 10.0 μg/ml HEL)/APC for 2 days by Western blot analysis. C, D10.G4.1 Th2 cells were transfected with a plasmid expressing the firefly luciferase gene under the control of the SOCS3 promoter (pGL3-SOCS3p), Gfi-1 cDNA mammalian expression vector (pcDNA-Gfi-1), and pRL-TK plasmid, which encodes Renilla luciferase as described under “Experimental Procedures.” Control cells were transfected with pcDNA vector alone or the pGL3-basic reporter plasmid (pGL3-Vector). Histograms represent SOCS3 promoter activity expressed in relative light units.
pathways involve activation of tyrosine kinases, we have used a model of forced SOCS3 expression to examine whether SOCS3 contributes to the regulation of both or either pathway. We show that significant increase in the steady-state levels of SOCS3 inhibits TCR-induced proliferation whereas depletion of SOCS3 levels by the antisense SOCS3 construct promotes proliferation of T-helper cells (Fig. 3E). Although the D10.G4.1 Th2 cells used in this study have obligatory requirement of exogenous IL-2 for growth, we are able to maintain the D10.G4.1 Th2 cells transfected with the antisense SOCS3 cDNA for 4 days in culture without exogenous IL-2, indicating that SOCS3 also regulates cytokine-induced proliferative responses and survival of T-helper cells. We further show that enhanced proliferation capacity observed when SOCS3 is transiently inhibited may be mediated, in part, by down-regulating expression of the cyclin-dependent kinase inhibitor p27kip1 (Fig. 4). These observations are in concert with reports showing that cytokine-stimulated T-cell proliferation is regulated by p27kip1 (27, 30).

To characterize mechanisms by which TCR activation down-regulates SOCS3 expression, we examined candidate genes that have been implicated in T-cell activation and proliferation. Of particular interest are members of the Gfi-1 family of transcriptional repressors that have multiple binding sites within the SOCS3 promoter (20). Gfi-1 is a transcriptional repressor that is preferentially expressed in lymphoid cells and is up-regulated in T-cells following Ag stimulation (19, 31). Gfi-1B is its cellular homologue that is preferentially expressed in erythroidopoietic organs such as bone marrow and spleen (20, 32), and it has been shown recently (20) to suppress erythropoietin-mediated SOCS gene expression in erythropoietin-responsive cell lines (20). These observations led us to hypothesize that Gfi-1 might be involved in repression of SOCS3 during T-cell activation. In this study, we show that T-helper cells express relatively low levels of Gfi-1. Antigenic stimulation induces its up-regulation, and the increase in Gfi-1 expression is temporally correlated with down-regulation of SOCS3 that is observed 48 h subsequent to T-helper cell activation. Taken together with our data showing that forced expression of Gfi-1 significantly inhibits SOCS3 promoter activity, these results suggest that the Ag-induced transient inhibition of SOCS3 transcription may be primary T-helper cell-mediated, in part, through Gfi-1. It is remarkable that fluctuation in the expression levels of Gfi-1 relative to SOCS3 parallels changes in IL-2 secretion during T-helper cell activation. For example, naïve cells that have relatively high constitutive SOCS3 expression secrete very little or no IL-2, while the observed decline in SOCS3 expression following 48 h of Ag stimulation coincides with increase in IL-2 secretion. Similar to Gfi-1, the increase in SOCS3 expression that occurs on day 4 of activation is associated with concomitant decline in IL-2 secretion, further underscoring the inverse relationship between the levels of SOCS3 expression and IL-2 secretion in differentiating T-helper cells (Table I, 96 h stimulation). These observations imply that IL-2 and SOCS3 may have mutually antagonistic effects during T-cell activation and are in line with a previous report showing that Th2 cells with high constitutive SOCS3 expression do not secrete IL-2 whereas Th1 cells that secrete large amounts of IL-2 have low SOCS3 expression (17). As the steady-state level of the Gfi-1 protein is under feedback regulation by IL-2-induced signal transducers and activators of transcription 5 signaling pathway (33), the observed fluctuations in the levels of these proteins during T-cell activation can be understood in the context of a feedback regulatory loop involving TCR signaling, IL-2 secretion, SOCS3, and Gfi-1.}

An important attribute of the adaptive immune system is its ability to discriminate between innocuous and dangerous antigenic stimuli (34). The data presented in this study suggest that SOCS proteins, particularly SOCS3, may regulate an important checkpoint that prevents inappropriate activation of T-helper cells or secretion of cytokines that underlie pathogenic mechanisms of autoimmune and allergic diseases. Thus, SOCS3 may function in vivo in concert with other proteins to maintain quiescent state of the lymphocytes. Induction of transient inhibition of SOCS3 expression by TCR signals of requisite strength and duration appear to be essential in allowing activation of quiescent T-helper cells.

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