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CUTTING EDGE

Cutting Edge: Suppressor of Cytokine Signaling 3 Inhibits Activation of NFATp

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Recent studies have suggested that signaling initiated by the activation of Ag receptors and signaling activated through cytokine receptors may be regulated by a common set of inhibitory proteins. Suppressor of cytokine signaling 3 (SOCS-3), which has previously been demonstrated to inhibit cytokine signaling, is induced on TCR ligation. Overexpression of SOCS-3 can inhibit transcription driven by the IL-2 promoter in response to T cell activation. This inhibitory activity correlates with the suppression of calcineurin-dependent dephosphorylation and activation of the IL-2 promoter binding transcription factor, NFATp. Infection of primary murine T cells with a retrovirus encoding SOCS-3 blocks their IL-2 production in response to activation. Interestingly, SOCS-3 was found to coimmunoprecipitate with the catalytic subunit of calcineurin. These studies suggest that SOCS-3 may regulate T cell function as part of a negative feedback loop. *The Journal of Immunology,* 2002, 168: 4277–4281.

R egulation of T cell activation is critical for the maintenance of immune homeostasis and the prevention of autoimmune disease (1). This homeostatic regulation can be implemented at several levels. Regulation of T cell activation at the level of cell-cell interaction involving negative regulatory receptors such as CTLA-4 and Fas is well-characterized (1). Much less is known about the negative regulation of T cell activation through TCR-mediated signaling pathways. Mechanisms involved in this regulation include recruitment of the inhibitory protein tyrosine kinase Csk (2), and protein tyrosine phosphatases such as Src homology 2-containing protein tyrosine phosphatase (SHP)[3]-1

and SHP-2 (3, 4). Interestingly, the SHP-1 and SHP-2 phosphatases also regulate cytokine receptor signaling (5). There are many similarities between TCR-mediated signaling and cytokine receptor-mediated signaling. Early events in both cases involve clustering of receptor subunits and the activation of tyrosine kinases. The most well-studied tyrosine kinases activated by cytokine receptors are the Janus kinase (JAK) family (6), whereas TCR ligation leads to activation of the Src family kinases, followed by activation of ZAP70 (7). Activated tyrosine kinases proceed to phosphorylate receptor chains and associated scaffolding proteins, which then bind further downstream effectors. One of the most well-characterized downstream effectors of TCR-mediated signal transduction is calcineurin, a calcium-dependent serine/threonine phosphatase that dephosphorylates and activates NFAT on TCR ligation (8). Important downstream effectors of cytokine receptor activation include the STAT family of transcription factors (6).

In addition to protein tyrosine phosphatase recruitment, another mechanism in the regulation of cytokine signaling involves members of the suppressor of cytokine signaling (SOCS) gene family (9–11). SOCS proteins have all been shown to inhibit cytokine signaling by interfering with the JAK-STAT pathway (12). The existence of parallels between cytokine receptor and TCR-mediated signaling has led to interest in the potential involvement of SOCS family members in TCR-mediated signaling. Cytokine-induced Src 2 homology-containing protein (CIS) has been shown to be induced by TCR stimulation and to enhance TCR-mediated mitogen-activated protein kinase activation, possibly through its interaction with protein kinase C-θ (13). Although the effects of SOCS-1 on TCR signaling remain unclear, SOCS-1 has recently been shown to inhibit the activation of NFAT in 293T cells expressing CD8 and Syk (14). Whether SOCS-3 plays a role in the regulation of TCR-mediated signaling has not previously been reported. In this report, we show that SOCS-3 is induced in T cells on TCR ligation and can inhibit TCR-mediated activation of the IL-2 promoter. Furthermore, SOCS-3 is shown to interact with calcineurin and inhibit the activation of NFATp in response to calcium signaling. Our results suggest a role for SOCS-3 in the regulation of NFATp activity in T cells.

Materials and Methods

Cell culture and transfection

Isolation and culture of primary CD4^{+} and Jurkat T cells were performed as previously described (15). Jurkat cells were transfected by electroporating Transfected cells were selected by resistance to G418 (Life Technologies, Gaithersburg, MD). 293T cells were transfected by the calcium phosphate method.

[3] Abbreviations used in this paper: SHP, Src homology 2-containing protein tyrosine phosphatase; HA, hemagglutinin; JAK, Janus kinase; SOCS, suppressor of cytokine signaling; CIS, cytokine-induced Src homology 2-containing protein; GFP, green fluorescent protein.

Intracellular cytoplasmic staining

Staining for intracellular PE-conjugated rat anti-IL-2 (BD PharMingen, San Diego, CA) was performed as previously described (16).

EMSA

The probe used was derived from the distal NFAT-binding site from the human IL-2 promoter; its sequence is 5′-GGAGGAAAAACTGTT TCATACAGAAGGCGT-3′. Double-stranded oligonucleotides were prepared and labeled by filling in recessed ends with Klenow enzyme (New England Biolabs, Beverly, MA). EMSA was performed as described previously (16, 17).

Immunoprecipitation, Western blotting, and Northern analysis

Cell extracts were made as described (18). Extracts were precleared with normal mouse IgG or normal rabbit IgG before incubation with NFATp antisera, hemagglutinin (HA) antisera, or SOCS-3 antisera (Santa Cruz Biotechnology, Santa Cruz, CA). Antisera used in Western blotting included NFATp, HA, PP-2Bβ (calcineurin), SOCS-3, (Santa Cruz Biotechnology), and Xpress (Invitrogen, San Diego, CA). Northern blotting was performed as described (16) using a SOCS-3 cDNA fragment.

GST precipitations

Plasmid-encoding GST-SOCS-3 fusion protein was constructed by cloning SOCS-3 into the *Bam*HI site of pGEX-3X (Amersham Pharmacia Biotech, Piscataway, NJ). GST and GST-SOCS-3 proteins were generated as described (19). Precipitation experiments were performed using beads coupled to 15 μg GST or GST-SOCS-3.

Retroviral infections

Infections were performed as previously described (20).

Results

TCR-regulated SOCS-3 expression

Several cytokines can induce SOCS-3 mRNA (9, 21). To examine the effects of TCR stimulation on SOCS-3 expression, RNA from purified murine T cells stimulated with anti-CD3 was examined. SOCS-3 mRNA levels were increased by stimulation with anti-CD3, reaching peak levels after 8 h of stimulation and remaining elevated for at least 24 h (Fig. 1). SOCS-3 induction by TCR ligation is less rapid and of much greater duration than induction by cytokines.

Inhibition of IL-2 promoter activity by SOCS-3

To investigate the effects of SOCS-3 expression on signaling downstream of the TCR, Jurkat cells were cotransfected with a luciferase reporter driven by the proximal IL-2 promoter and either SOCS-3 or a vector control. SOCS-3 inhibited the activation of the

IL-2 promoter by PMA and ionomycin (Fig. 2*A*). Further cotransfection experiments using a luciferase reporter driven by three copies of the distal NFAT/AP-1 site from the IL-2 promoter revealed the ability of SOCS-3 to inhibit transcriptional activation driven by this element (Fig. 2*B*). In contrast, SOCS-3 had no effect on the PMA/ionomycin induced activation of a luciferase reporter driven by multimerized AP-1 binding sites (Fig. 2*C*) or a multimerized NF-κB binding site (data not shown). The results of these transient transfection experiments suggest that SOCS-3 suppresses NFAT-dependent transcriptional activation. Furthermore, the ability of SOCS-3 to block reporter activity induced by PMA and ionomycin suggests that it acts downstream of the initial tyrosine kinase activity of *lck* and ZAP70.

SOCS-3 inhibits NFATp dephosphorylation

To further study the effects of SOCS-3 on NFAT function, Jurkat cells constitutively expressing SOCS-3 were generated. Jurkat
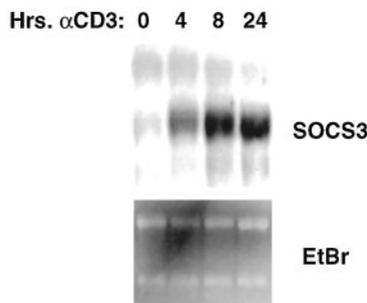
FIGURE 1. Changes in SOCS-3 mRNA levels on TCR ligation and effects of SOCS-3 on NFAT-mediated transcription. RNA was isolated from splenic T cells of C57BL/6 mice stimulated with plate-bound anti-CD3 (αCD3) for the amount of time indicated. The RNA was then subjected to Northern blot analysis with probe specific for SOCS-3 (*top*). Ethidium bromide (EtBr) staining of RNA gel demonstrates equal loading of lanes (*bottom*).
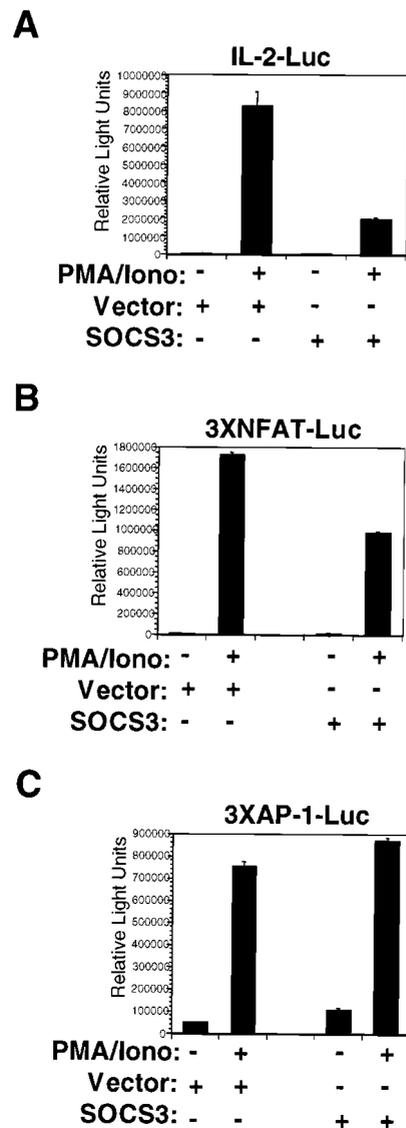
FIGURE 2. SOCS-3 suppresses NFAT-mediated transcriptional activation. *A,* Jurkat cells were transfected with an IL-2-Luc reporter (20 μg) and either pcDNA3 or pcDNA3-SOCS-3 (10 μg). Twelve hours after transfection, cells were cultured with or without PMA (100 nM) and ionomycin (Iono; 2 μM) for 8 h, after which cells were harvested and analyzed for luciferase activity. Transfections were repeated as above using a 3XNFAT-Luc reporter (*B*) and a 3XAP-1-Luc reporter (*C*).

cells expressing SOCS-3 were deficient in their ability to produce IL-2 as compared with Jurkat cells transfected with vector alone when stimulated with PMA and ionomycin (Fig. 3A). When Jurkat cells expressing SOCS-3 were stimulated with anti-CD3, the pattern of tyrosine phosphorylation seen in whole cell extracts was similar to that observed in cells transfected with empty vector (data not shown), again suggesting that SOCS-3 does not interfere with the activation of tyrosine kinases through the TCR. To determine whether the overexpression of SOCS-3 altered NFAT activation in these cells, NFAT was investigated directly by EMSA. Nuclear extracts from stimulated Jurkat cells expressing SOCS-3 show reduced binding to the distal NFAT site in the IL-2 promoter. These data demonstrate that overexpression of SOCS-3 can inhibit NFAT activation and IL-2 production in Jurkat cells.

Immunoblotting demonstrated no significant differences in levels of $NFAT_p$ were seen between Jurkat cells transfected with SOCS-3 and those transfected with empty vector (data not shown). Because the nuclear entry of NFAT depends on dephosphorylation of multiple serine/threonine residues in response to sustained increases in intracellular calcium, the dephosphorylation of NFATp induced by a 2-min stimulation with ionomycin was examined. Dephosphorylation of NFATp leads to a 10- to 20-kDa decrease in its apparent molecular mass by immunoblot (22, 23). NFATp was immunoprecipitated from stimulated or unstimulated stably transfected Jurkat cells and analyz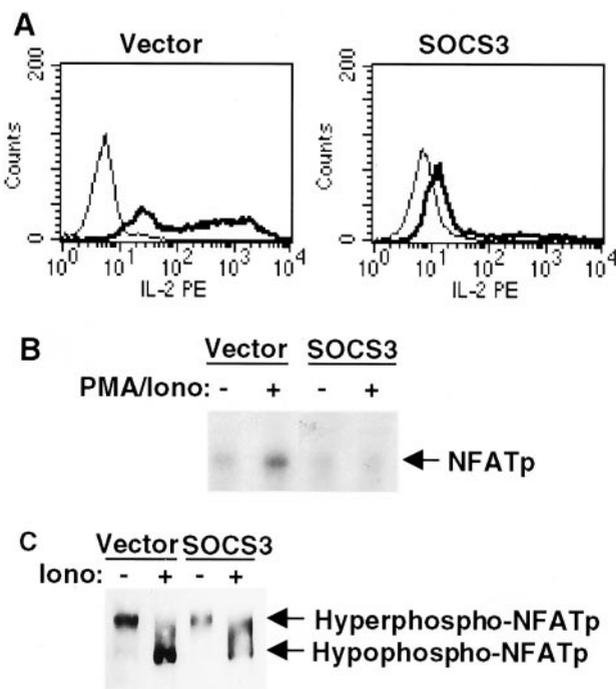ed by immunoblotting for a decrease in apparent molecular mass. Less efficient dephosphorylation of NFATp was observed in Jurkat cells expressing SOCS-3 than in Jurkat cells transfected with empty vector (Fig. 3C). This result suggests that SOCS-3 interferes with the activation of NFATp by inhibiting its dephosphorylation.

Interaction of SOCS-3 with calcineurin

The observation that SOCS-3 inhibited NFATp dephosphorylation induced by ionomycin raised the possibility that SOCS-3 could interact with NFATp or calcineurin. To determine whether SOCS-3 interacts with these molecules, whole cell extracts from 293T cells transfected with calcineurin, NFATp, or empty vector were incubated with agarose beads coupled to GST or a GST-SOCS-3 fusion protein. Although there is a slight interaction between calcineurin and beads coupled to GST, a much stronger interaction was observed between calcineurin and beads coupled to GST-SOCS-3 (Fig. 4A). The association of calcineurin with beads coupled to GST-SOCS-3 was also much stronger than that seen between NFATp and beads coupled to GST-SOCS-3 (Fig. 4A).
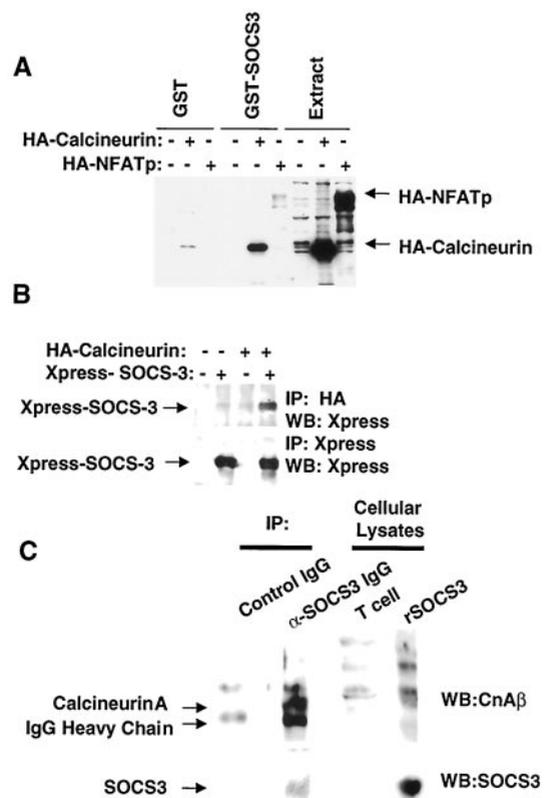

FIGURE 3. SOCS-3 inhibits NFATp activation. A, Intracellular cytoplasmic staining for IL-2. Jurkat cells stably transfected with pcDNA3 or pcDNA-SOCS-3, as indicated, were left unstimulated (thin line) or stimulated for 20 h with PMA and ionomycin (thick line). After stimulation, samples were fixed, permeabilized, and stained with PE-conjugated rat anti-murine IL-2 (BD PharMingen) for analysis by flow cytometry. B, Examination of NFAT activation by EMSA. Nuclear extracts were prepared from stably transfected Jurkat cells unstimulated or stimulated for 4 h with PMA (100 nM) and ionomycin (2 μM). Extracts were assayed for binding to the distal NFAT-binding site of the human IL-2 promoter. C, Analysis of NFATp dephosphorylation. Stably transfected Jurkat cells were unstimulated or stimulated with ionomycin (2 μM) for 2 min. Whole cell extracts were immunoprecipitated with antisera to NFATp and analyzed by immunoblot for NFATp.

FIGURE 4. Interaction of SOCS-3 and calcineurin. A, Agarose beads coupled to GST or GST-SOCS-3 were incubated with extracts from 293T cells untransfected or transfected with HA-tagged calcineurin or HA-tagged NFATp. Proteins associated with the beads were analyzed by immunoblot for HA. Extract lanes represent 10% of total input. B, 293T cells were untransfected or transfected with HA-tagged calcineurin, Xpress-tagged SOCS-3, or both, as indicated. Whole cell extracts were immunoprecipitated (Ip) with antisera to HA (*top*) or Xpress (*bottom*) and immunoblotted for Xpress. C, T cells extracted from the spleens of eight C57B6 mice were purified for CD4+ cells and stimulated with plate-bound anti-(α-) CD3 and anti-CD28 Abs followed by lysis. The resulting 7.5 mg of extract were immunoprecipitated with either control rabbit IgG or SOCS-3 antisera and immunoblotted for either calcineurin or SOCS-3. Extract lane represents <2% of total input. Recombinant protein produced by transfecting 293T cells with a SOCS cDNA was loaded as a positive control. WB, Western blot; CnAβ, calcineurin Aβ.
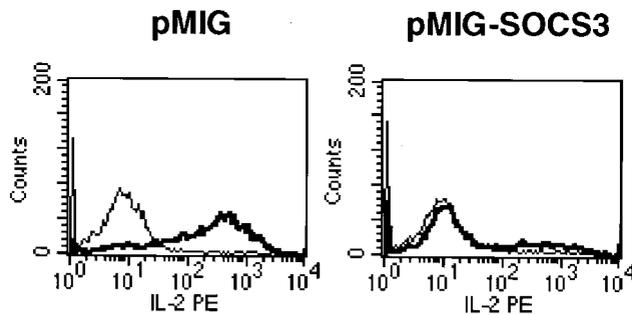
pMIG　　　　　pMIG-SOCS3

FIGURE 5. Intracellular cytoplasmic staining for IL-2 in primary T cells. CD4$^+$ T cells were isolated from lymph nodes of C57BL/6 mice and stimulated with plate-bound anti-CD3 and anti-CD28. Twenty-four and 48 h after stimulation, cells were transduced with either pMIG or pMIG-SOCS-3 retroviruses. Five days after stimulation, cells were unstimulated (thin line) or stimulated with PMA (100 nM) and ionomycin (2 μM) for 4 h (thick line). After stimulation cells were stained with CyChrome-conjugated anti-CD4, fixed, and stained for intracellular IL-2 as in Fig. 2*A*. Cells expressing both CD4 and GFP by flow cytometry were analyzed for intracellular IL-2 content.

The reverse interaction of SOCS-3 with calcineurin was tested by transfecting 293T cells with combinations of SOCS-3, calcineurin, and empty vector. SOCS-3 coimmunoprecipitated with calcineurin only in cells transfected with both genes (Fig. 4*B*). Furthermore, we investigated the interaction between SOCS-3 and calcineurin in vivo. Primary murine splenocytes were stimulated with plate-bound Abs to CD3 and CD28 for 48 h to induce SOCS-3, followed by lysis and immunoprecipitation. We found that SOCS-3 interacts with calcineurin Aβ in stimulated primary T cells.

SOCS-3 inhibits IL-2 production in primary CD4$^+$ T cells

Given that both the interaction of SOCS3 with calcineurin and the initial observation of SOCS-3 mRNA modulation by anti-CD3 was made in primary murine T cells, the ability of SOCS-3 to alter NFAT-dependent responses in these cells was examined. CD4$^+$ T cells isolated from lymph nodes of C57BL/6 mice were infected with retroviruses encoding either SOCS-3 and green fluorescent protein (GFP) or GFP alone. Three days after infection, the cells were stimulated for 4 h with PMA/ionomycin and analyzed for IL-2 production by intracellular cytoplasmic staining. Although PMA/ionomycin induced a marked activation of IL-2 synthesis in cells expressing GFP alone, this activation was greatly reduced in cells also expressing SOCS-3 (Fig. 5). The failure of T cells expressing SOCS-3 to synthesize IL-2 resembles that seen in T cells from transgenic mice expressing a dominant negative NFAT (24).

Discussion

Although the above results are intriguing, the observed inhibition of NFATp activation by SOCS-3 relies on overexpression of SOCS-3 in T cells. Therefore, it remains unclear whether SOCS-3 plays a role in regulating NFATp activation in vivo. Encouraging results in this regard include the increase in SOCS-3 mRNA on TCR ligation and the inhibition of activation-induced IL-2 production on SOCS-3 overexpression in primary murine T cells. *SOCS-3* gene deletion in mice results in severe erythrocytosis and embryonic lethality at days 12–16 (25). *SOCS-3*-deficient T cells generated by reconstitution of JAK3-deficient mice or lethally irradiated wild-type mice with *SOCS-3*-deficient fetal liver were not grossly abnormal (25). Further analysis of T cell development and function in these chimeric mice will help clarify the role of SOCS-3 in vivo.

The ability of SOCS-3 to associate with calcineurin leads to the question of whether SOCS-3 affects the phosphatase activity of

calcineurin. Calcineurin phosphatase activity toward an in vitro labeled peptide from the RII subunit of protein kinase A (26) was similar in extracts from Jurkat cells stably transfected with SOCS-3 and extracts from Jurkat cells transfected with empty vector (data not shown). Further phosphatase assays using extracts from 293T cells transfected with calcineurin demonstrated that recombinant GST-SOCS-3 does not inhibit the dephosphorylation of in vitro labeled peptide by calcineurin (data not shown). This inability of SOCS-3 to block calcineurin phosphatase activity toward an in vitro labeled peptide substrate leaves open the question of the mechanism of SOCS-3 in the inhibition of NFATp activation. One possibility is that SOCS-3 alters the interaction between NFATp and calcineurin by competing with NFATp for a binding site on calcineurin. It is also possible that SOCS-3 simultaneously associates with calcineurin and a serine/threonine kinase, bringing a kinase capable of phosphorylating NFATp to the NFATp-calcineurin complex. In support of this possibility, SOCS-3 has been shown to interact with the Pim family of serine/threonine kinases (B. Vuong and P. Rothman, unpublished data), and Pim family kinases can phosphorylate NFAT in vitro (27, 28). In the case of cytokine signaling, CIS, SOCS-1, and SOCS-3 are all negative regulators of JAK-STAT signaling (12). In contrast, CIS, SOCS-1, and SOCS-3 have divergent effects on TCR-mediated signals.

The observed increases in SOCS-3 mRNA levels in T cells activated through the TCR suggest the involvement of SOCS-3 in a classical feedback loop of TCR-mediated signal transduction. A recent report showing the ability of SOCS-3 to inhibit IL-2 signaling in lymphocytes adds an interesting dimension to the loop, because IL-2 cooperates with TCR-mediated signals to stimulate T cell proliferation (29). SOCS-3 may have a dual role in regulating T cell responses, inhibiting the activation of both NFAT by calcineurin and JAK1 by the IL-2 receptor.

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