A Role of Suppressor of Cytokine Signaling 3 (SOCS3/CIS3/SSI3) in CD28-mediated Interleukin 2 Production

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Abstract

Suppressor of cytokine signaling (SOCS)3 has been characterized as a negative feedback regulator in cytokine-mediated Janus kinase signal transducer and activator of transcription signaling. However, this study shows that T cells from transgenic mice expressing SOCS3 exhibit a significant reduction in interleukin (IL)-2 production induced by T cell receptor cross-linking when T cells are costimulated with CD28. Decreased protein expression in SOCS3$^{+/-}$ mice enhanced CD28-mediated IL-2 production, clearly indicating the correlation between expression level of SOCS3 and IL-2 production ability. The SOCS3 protein interacted with phosphorylated CD28 through its SH2 domain but not the kinase inhibitory region. In addition, a point mutation in the SOCS3 SH2 domain attenuated the inhibition of CD28 function in IL-2 promoter activation. Committed T helper (Th)2 cells exclusively expressed SOCS3 and production of Th2 cytokines, such as IL-4 and IL-5, was much less dependent on CD28 costimulation compared with interferon γ and IL-2 production in Th1 cells. Consistent with this notion, the expression level of SOCS3 in early T cell activation influenced the ability of IL-2 production induced by CD28 costimulation. Therefore, the SOCS3 may play an alternative role in prohibiting excessive progression of CD28-mediated IL-2 production.

Key words: CD28 • costimulation • IL-2 production • SOCS • T cell activation

Introduction

T cell activation is triggered by the interaction between peptide-bound MHC molecules on APCs and TCR on T cells. An additional receptor–ligand interaction results in the full activation of T cells by providing costimulatory signals. One prominent T cell costimulatory receptor that has been studied extensively is the CD28 molecule (1, 2). CD28 is expressed on the majority of mature T cells and binds to B7-1 (CD80) and B7-2 (CD86) that are either induced or constitutively expressed on APCs. The CD28 binding to those ligands results in T cell costimulation whereas the binding of cytotoxic T lymphocyte–associated antigen 4, a structurally homologous receptor to CD28, results in the inhibition of T cell activation (3–5). CD28 co-stimulation plays an essential role on IL-2 production in primary T cell activation, driving subsequent cell cycle progression and cell commitment. In its absence T cells become anergic (6).

The cytokine signaling is known to be regulated by a negative feedback regulator called suppressor of cytokine signaling (SOCS),* the cytokine-inducible SH2-containing protein (CIS), and signal transducer and activator of transcription (STAT)-induced STAT inhibitors family (7–9). Members of the SOCS family have been identified due to their marked homology on the SH2 domain and a unique

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Abbreviations used in this paper: CIS, cytokine-inducible SH2-containing protein; ERK, extracellular signal–regulated kinase; Grb2, growth factor receptor–bound protein; GST, glutathione S-transferase; JAK, Janus kinase; KIR, kinase inhibitory region; NF, nuclear factor; PI3-K, phosphatidylinositol-3-kinase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; Tg, transgenic.

conserved motif referred to as the SOCS box (10). SOCS3 has been characterized as a protein related to CIS1 and SOCS1 and the expression is transiently induced in a variety of immune and inflammatory situations by a wide variety of cytokines, including IFN-γ, IL-2, IL-3, IL-6, and IL-10 (7, 9, 11). In vitro studies have proposed roles for SOCS3 in growth hormone function (12), IL-2–dependent T cell proliferation (13), leptin (14), IL-10 (15), and IL-11 (16) signaling. Both SOCS1 and SOCS3 inhibit Janus kinase (JAK) tyrosine kinase activity. SOCS1 directly binds to the activation loop of JAKs through the SH2 domain whereas SOCS3 binds to the cytokine receptors. These two molecules contain a similar kinase inhibitory region (KIR) at the NH_2 terminus that is essential for JAK inhibition (17, 18). We proposed that KIR interacts with the region close to the catalytic groove of the JAK2 kinase domain, thereby preventing the access of substrates to the catalytic pocket. SOCS3 has been shown to bind to Y757 of gp130 as well as to Y401 of the erythropoietin receptor, which are the same binding sites for SHP-2 (19, 20, 21).

Disruption of the SOCS3 gene was shown to result in an embryonic lethality associated with erythrocytosis (22) and defects in the placenta (23). Major expression of SOCS3 mRNA has been found in the spleen and thymus, suggesting the importance of SOCS3 in T cell function. T cell populations that reconstituted in JAK-deficient mice with fetal liver and the reconstituted T cells were not grossly abnormal in T cell development and responsiveness against cytokine stimulation (22). However, the alternative function of SOCS3 in the T cell activation process still remains undetermined. Thus, we studied a role of SOCS3 in T cell receptor complex–related signaling.

The tyrosine phosphorylation of CD28 regulates the binding of the lipid/serine kinase, phosphatidylinositol-3-kinase (PI3-K), and the adaptor protein, growth factor receptor–bound protein (Grb2), to the cytoplasmic region of CD28 (24–28). PI3-K is a heterodimer composed of a dimer of a p85 regulatory subunit and a p110 catalytic subunit. Phosphorylation of CD28 on tyrosine residue 189, present in a YMNM motif, has been shown to provide a binding site for the SH2 domain of the p85 regulatory subunit (24). The binding of PI3-K is thought to be crucial for CD28-mediated IL-2 production. CIS1 and SOCS3 have a similarity to the PI3-K in the SH2 domain sequence (18). Therefore, we speculate that SOCS3 plays an alternative role as a negative regulator for CD28 costimulation. We have consistently demonstrated that the IL-2 production ability induced by CD28 costimulation was tightly correlated with the expression level of SOCS3. This inhibitory mechanism seems to be distinct from that seen for cytokine signaling. We further studied the mechanisms regulating the alternative inhibitory functions of SOCS3 for CD28-mediated signaling.

Materials and Methods

Mice. The myc-tagged SOCS3 transgenic (Tg) mice under the control of the lck proximal promoter and the intronic en-

hancer from the Ig heavy chain locus (Eμ) promoter were generated and backcrossed into C57BL/6 mice. Heterozygous SOCS3 mice were obtained from Jim N. Ihle (St. Jude Children's Research Hospital, Memphis, TN). DO11.10 Tg mice and IL-4R–deficient mice on BALB/c background were provided by Kenneth Murphy (Washington University, St. Louis, MO) and Frank Brombacher (University of Cape Town, Cape Town, South Africa). DO11.10 Tg mice and IL-4R–deficient mice were additionally backcrossed into BALB/c mice for >10 generations.

Proliferative Responses and Preparation of $CD4^+$ T Cells. Whole spleen cells were stimulated with various concentrations of Con A for 36 h and pulse labeled with 1 mCi of [³H]thymidine for an additional 8 h. The proliferative responses were measured as [³H]thymidine incorporation. The enriched $CD4^+$ T cells were prepared from spleen cells by incubation with anti-CD8 mAb (3–155) followed by incubation with anti–mouse Ig–coated plates to eliminate B and $CD8^+$ T cells.

Measurement of IL-2 Concentration by ELISA. The enriched $CD4^+$ T cells were stimulated with plate-bound anti-TCR mAb (H57-597) in the presence or absence of anti-CD28 mAb (PV-1; reference 29) for 24 h. The culture supernatants were harvested and cytokine concentration in the supernatant was measured as previously described (30). In brief, the supernatants were applied on the plastic plate coated with anti–IL-2 mAb (JES6-1A12). After washing, the plate was probed with biotin-conjugated anti–IL-2 mAb (JES6-5H4) and horseradish peroxidase–conjugated streptavidin (Zymed Laboratories) and developed with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; Kirkegaard & Perry Laboratories). The 405-nm absorbance was measured by spectrophotometer (Bio-Rad Laboratories).

Nuclear Extracts and Electrophoretic Mobility Shift Assay. Extractions of nuclear proteins and electrophoretic mobility shift assays were performed as previously described (31). The splenic T cells were cultured with TCR or TCR/CD28 for 36 h. After washing with PBS, hypotonic buffer was added to the cell pellet and incubated on ice for 10 min. The cell membranes were disrupted with 0.15% NP-40. The nuclei were centrifuged at 2,000 rpm for 10 min and the pellets were resuspended in extraction buffer containing 0.3 M $(NH_4)_2SO_4$. After incubation at 4°C for 30 min, the nuclear proteins were centrifuged at 100,000 rpm for 10 min and precipitated with 1.5 M $(NH_4)_2SO_4$. The nuclear proteins were incubated with 10^4 cpm end-labeled oligonucleotide probe for 30 min at room temperature in binding buffer. The samples were loaded on a 4% polyacrylamide gel and run in low ionic strength buffer. The sequences of oligonucleotide probe for the nuclear factor (NF)-κB is AGTTGAGGG-GACTTTCCCAGGC.

Northern Hybridization. Total RNA was isolated by the TRIzol reagent (GIBCO BRL) and separated on 1.0% agarose gels containing 2.4% formaldehyde. RNA was transferred to nylon membranes and then the membranes were hybridized with digoxigenin-labeled riboprobes and visualized by alkaline phosphatase–leveled anti–digoxigenin antibody (Roche Diagnostic).

Reagent and Immunoblot Analysis. Total cell extracts were prepared from tissue homogenized in 50 mM Tris-HCl, pH 8.0, 0.5% NP-40, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM PMSF with a protease inhibitor cocktail (Sigma-Aldrich). The samples were resolved on SDS-PAGE and the proteins were detected by immunoblotting. Antibodies against murine extracellular signal–regulated kinase (ERK)2, myc, and CD28 were purchased from Santa Cruz Biotechnology, Inc. Tyrosine-phosphorylated ERKs were detected by anti-

The Journal of Experimental Medicine

pERK antibodies (Promega). Antibodies against SOCS3 were raised in rabbit using a synthetic peptide.

Binding of SOCS3 to Glutathione S-transferase (GST) Protein In Vitro. GST-CD28 construct was provided by Takashi Saito (Chiba University, Chiba, Japan). GST-CD28 in pGEX-4T-1 (Amersham Biosciences) were transformed into *Escherichia coli* BL21(DE3) strain or TKB1 strain, which is a tyrosine kinase derivative of the BL21(DE3) (Stratagene). GST fusion protein was induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside for 2 h and immobilized on glutathione-Sepharose 4B (Amersham Biosciences). Myc-tagged SOCS3 was transfected into HEK293 cells and the transfected cells were extracted after 24 h of transfection. Cell extracts were incubated with 20 μl (50% vol/vol) of GST-Sepharose for 1 h at 4°C. After washing twice with lysis buffer, the precipitates were analyzed by immunoblotting.

Preparation of Th1 and Th2 Cells. DO11.10 Tg spleen cells were incubated with anti-CD8 mAb (3–155) at 4°C and the cells were incubated on plate-coated anti–mouse Ig to eliminate B and CD8$^+$ T cells to isolate CD4$^+$ T cells. The CD4$^+$ T cells were stimulated with OVA antigenic peptide (residues 323–339; ISQAVHAAHA EINEAGR; BEX Corporation) in the presence of irradiated BALB/c APCs. The induction of Th1 and Th2 cells was controlled by the addition of either 10 unit/ml mouse IL-12 plus anti–IL-4 mAb or 100 unit/ml mouse IL-4 plus anti–IL-12 mAb, respectively.

Transfection and Luciferase Assay. 5×10^6 Jurkat cells were cotransfected with 5 μg IL-2 reporter construct (31), 2 μg pSV2α placental alkaline phosphatase, 5 μg pcDNA3 mouse CD28, and 5 μg pcDNA3 myc SOCS3 constructs by electroporation (32). The pSV2α phosphatase plasmid was used for normalizing the transfection efficiency. The transfected cells were stimulated for 12 h with 10 μg/ml anti–human CD3 mAb (OKT3), a combination of anti-CD3 and anti–mouse CD28 (PV-1) mAbs, or a combination of 50 ng/ml PMA plus 1 μM ionomycin. After 12 h, the cells were harvested and divided into two groups. 20% of the cells were then used for the measurement of alkaline phosphatase activity. The rest of the cells were used for the measurement of the luciferase activity. The emitted luciferase light in substrate solution (Promega) was measured with a luminometer (Analytical Luminescence Laboratory).

Results

SOCS3 Negatively Regulated CD28-mediated IL-2 Production. Here, we attempted to study the role of SOCS3 in TCR-related responses using Tg strains of mice expressing WT SOCS3 driven by lck-Eμ promoter. All four Tg lines were indistinguishable from control littermates in body size, weight, and behavior. Thymocytes and splenic T cells from Tg mice exhibited similar expression profiles of several T cell markers (CD4, CD8, TCRβ, CD28, CD25, CD69, CD44, and CD62L) compared with normal littermate mice, although the number of thymocytes and splenic T cells was slightly reduced in Tg mice (unpublished data). Protein expression from the myc-tagged transgene was ~5–10 times higher than that of endogenous SOCS3 (unpublished data).

To study the proliferative responses of peripheral T cells, splenocytes were stimulated with T cell mitogen, Con A. As shown in Fig. 1 A, spleen cells from SOCS3 Tg mice showed a marked reduction in the proliferative responses

to approximately half that seen in littermate mice. This result raised the possibility that SOCS3 was implicated in the negative regulation of TCR-mediated signaling. Thus, IL-2 production in CD4$^+$ naive T cells was measured at various time points after stimulation with anti-TCR mAb in the presence or absence of CD28 costimulation. In control mice, the anti-TCR mAb-activated T cells expressed very low but detectable amounts of IL-2 and in conjunction with CD28 costimulation, IL-2 production was very strongly enhanced ~250 times. Interestingly, SOCS3 Tg T cells consistently exhibited less augmentation in CD28-mediated IL-2 production although the TCR–mediated IL-2 production was comparable to that of control littermate mice (Fig. 1 B). The augmentation rate in SOCS3 Tg was consistently less than half of that in the control mice (Fig. 1 B). A similar reduction was observed in the Tg mice expressing the mutant of SOCS3, F25A, which contains a point mutation in KIR that is a critical for the inhibition of cytokine signaling including IL-2 (unpublished data). F25A SOCS3 mutant inhibited CD28-mediated augmentation in IL-2 production (Fig. 1, B and C). This SOCS3-mediated inhibition could not be attributed to the alteration of CD28 expression levels because T cells from SOCS3 Tg exhibited comparable CD28 expression to control T cells (unpublished data). Therefore, we speculate that SOCS3 has an alternative role as a negative regulator of the CD28 costimulation signal and machinery of the negative regulation seems to be distinctive from that of the cytokine signaling.

Forced SOCS3 Expression Inhibits CD28-mediated NF-κB Activation. A recent report addressed the possibility that SOCS3 inhibits TCR signaling in the human Jurkat cell line via the binding to the active form of calcineurin (33). However, the alteration of SOCS3 expression did not affect the IL-2 production in response to TCR alone in SOCS3 Tg mice. Thus, we further explored the relevance of SOCS3 in TCR-mediated T cell responses and examined the cell surface expression of the T cell activation markers, CD25 and CD69. The up-regulation of these two molecules in activated T cells is mainly controlled by TCR-mediated signals. As shown in littermate control splenic T cells (Fig. 2 A), TCR stimulation fully activated CD25 and CD69 expression within 24 h, but CD28 signal did not show costimulation effect in CD25 and CD69 expression. T cells from SOCS3 Tg mice also exhibited full activation of CD25 and CD69 expression, supporting our speculation that SOCS3 has no inhibitory effect on the signaling events mediated by TCR activation.

CD28 can activate several signaling events that integrate those governed by TCR cross-linking. Thus, CD28 increases TCR-mediated ERK activation by Rap-1 inhibition (34). Next, we explored SOCS3 function on the ERK activation by TCR and CD28 stimulation. In littermate T cells, ERK1/2 phosphorylation was induced with TCR cross-linking and CD28 clearly enhanced ERK1/2 activation. However, unlike littermate T cells, SOCS3 Tg CD4$^+$ T cells showed ERK1/2 phosphorylation without the stimulation. The ERK1/2 phosphorylation was marginally
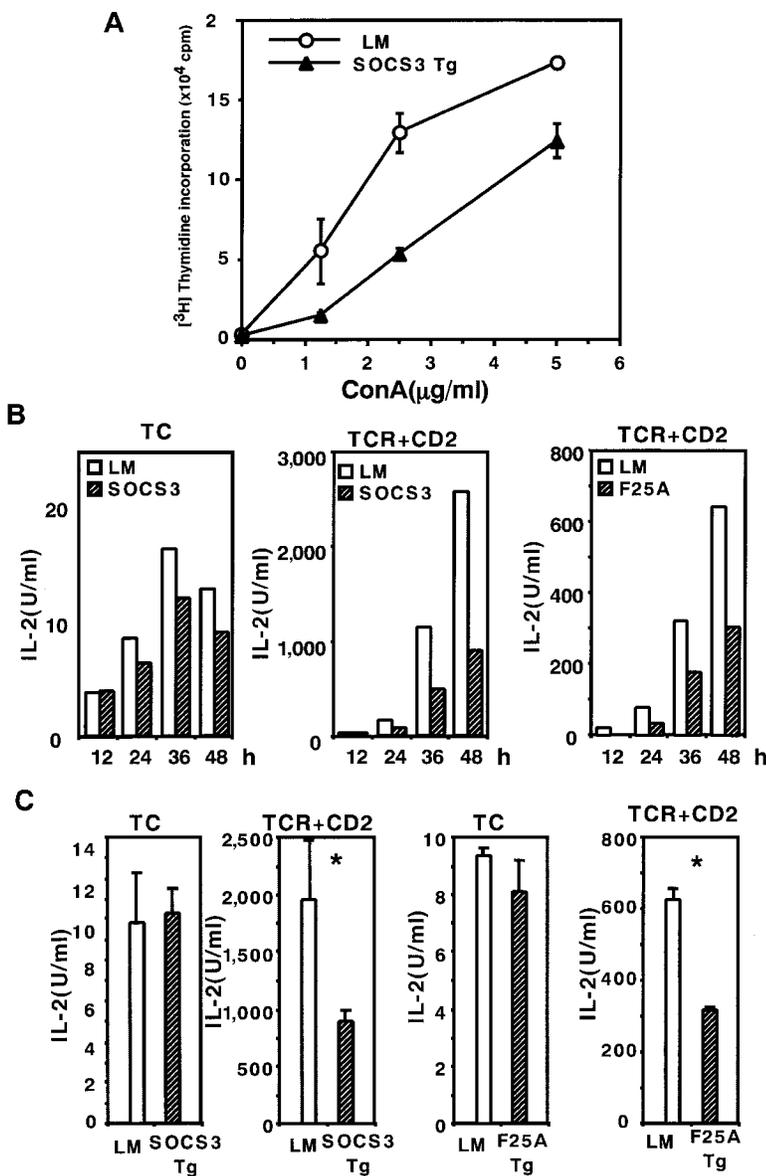
Figure 1. Impairment of Con A–induced T cell activation and CD28-induced IL-2 production in T cells from SOCS3 Tg mice. (A) Impairment of Con A–induced T cell activation in SOCS3 Tg mice. Whole spleen cells from control littermate (LM) or SOCS3 Tg mice were stimulated with various concentration of Con A for 36 h. Proliferative response was measured by [³H]thymidine incorporation in the last 8 h. (B) IL-2 production in T cells from control LM, WT SOCS3, and KIR mutant SOCS3 F25A Tg mice. The CD4⁺ T cells from control LM, SOCS3 Tg, and F25A Tg mice were stimulated with anti-TCR mAb or a combination of anti-TCR and anti-CD28 mAb. The culture supernatants were harvested at various time points and the amount of IL-2 was measured by ELISA. (C) IL-2 production at 48 h in the activated T cells from SOCS3 and F25A Tg. The mean ± SD shown was obtained from five mice. *, P < 0.05 versus control littermates.

enhanced by TCR cross-linking but not by CD28 costimulation (Fig. 2 B). These results indicated that constitutive expression of SOCS3 altered the phosphorylation status of ERK pathway in CD4⁺ T cells, perhaps because SOCS3 alone can partially enhance basal ERK activation levels (35), but this alteration might not influence the TCR-induced IL-2 production and expression of activation markers. Even under these circumstances, SOCS3 might have retained the inhibitory function for the CD28-mediated enhancement of ERK1/2 phosphorylation.

Maximum activation of the IL-2 gene is accomplished by the combination of TCR and CD28 costimulation. These two signals lead to the synergistic activation of NH₂-terminal kinase and NF-κB. The activated NF-κB is known to bind the CD28RE contained in the IL-2 promoter, leading to a marked up-regulation in IL-2 transcription. To study a role of SOCS3 in CD28-mediated NF-κB

activation, CD4⁺ T cells were stimulated with TCR cross-linking in the presence or absence of anti-CD28 mAb. The translocation of NF-κB into the nuclei was assessed by the binding to the putative sequence for NF-κB. As shown in Fig. 2 C, TCR cross-linking induced weak NF-κB nuclear translocation and CD28 signal was required for full activation of NF-κB in control mice. The T cells from SOCS3 Tg mice exhibited less augmentation in NF-κB nuclear translocation by CD28 costimulation, indicating that consistent with the IL-2 production, SOCS3 inhibited NF-κB activation induced by CD28 costimulation.
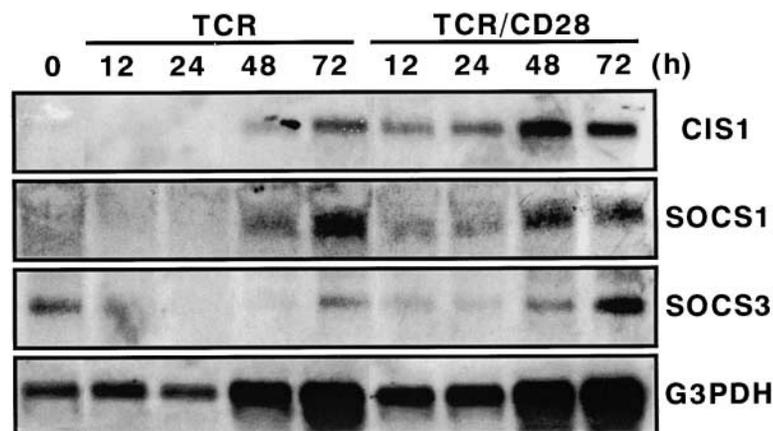
Induction of SOCS3 Expression during Primary T Cell Activation. Forced expression of SOCS3 inhibits the CD28 costimulation-mediated IL-2 production in early T cell activation, but it was still unclear whether a significant amount of SOCS3 are expressed during early T cell activation. Thus, we examined the expression of CIS-1, SOCS1, and
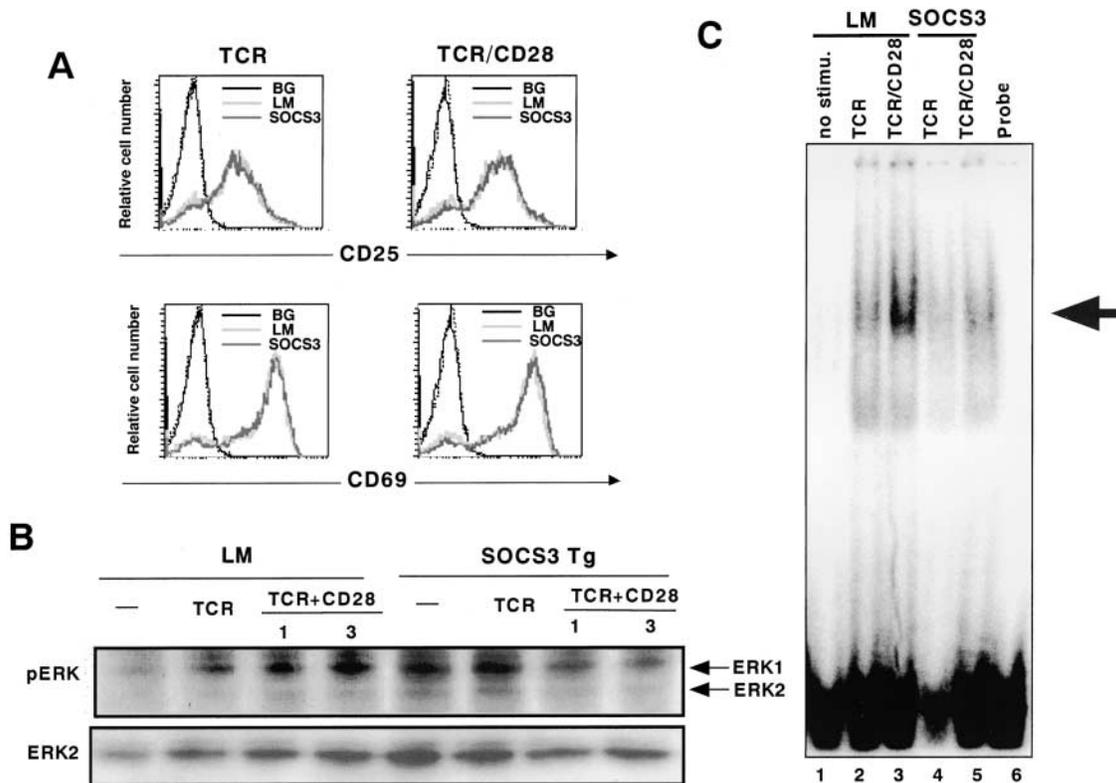
Figure 2. Effects of constitutive expression of SOCS3 on TCR and CD28-mediated signaling events. (A) Effects of constitutive expression of SOCS3 on CD25 and CD69 expression. The CD4$^+$ T cells from littermate (LM) and SOCS3 Tg mice were stimulated with plate-bound anti-TCR in the presence or absence of anti-CD28 for 48 h and then the expression of CD25 and CD69 were analyzed by flow cytometric analysis. (B) Effects of constitutive expression of SOCS3 on ERK activation. Naive splenic T cells were incubated with anti-TCR in the presence or absence of anti-CD28 mAb (1 and 3 μg/ml). Cell extracts were resolved by SDS-PAGE and immunoblotted with anti-pERK (top) or anti-ERK2 (bottom) antibody. (C) Effects of constitutive expression of SOCS3 on CD28-mediated NF-κB activation. Electophoretic mobility shift assays were performed with nuclear extracts from T cells unstimulated (lane 1), stimulated with anti-TCR (lanes 2 and 4), and anti-TCR plus anti-CD28 mAb (lanes 3 and 5). Arrow indicates the binding of NF-κB.

SOCS3 in the primary stimulation of naive T cells. CD4$^+$ T cells from BALB/c mice were activated with either TCR or TCR plus CD28 mAbs and mRNA expression was assessed by Northern blot analysis. The CIS-1 and SOCS1 expressions were also induced by a combination of TCR and CD28 costimulation and the maximum expression level was found within 48 h (Fig. 3). The expression profile of SOCS3 mRNA differed from that of CIS-1 and SOCS1

because resting CD4$^+$ T cells expressed a significant level of SOCS3 and the expression was rapidly decreased after TCR stimulation. The expression recovered after 48 h and reached the maximum level at 72 h when T cells were co-stimulated with anti-CD28 mAb (Fig. 3). Therefore, a significant amount of SOCS3 were expressed in the primary IL-2 production process and CD28 costimulation may play a role to rapidly enhance the expression level of SOCS3.


Figure 3. Induction of SOCS3 expression during early T cell activation. CD4$^+$ T cells from BALB/c mice were activated with either TCR or TCR plus CD28 mAbs and mRNA expressions of CIS-1, SOCS1, SOCS3, and G3PDH were assessed by Northern blot analysis at various time points after stimulation.

The Journal of Experimental Medicine

Increased CD28-mediated IL-2 Production in SOCS3 Heterologous Mice. In forced expression studies using Tg approaches it is difficult to fully interpret the physiological relevance of SOCS3. Thus, we next assess the CD28-mediated IL-2 production in SOCS3 heterologous mice. Naive CD4^{+} T cells did not exhibit detectable protein expression of endogenous SOCS3 and the protein expression only appeared after stimulation with anti-TCR and CD28 mAb. The protein expression levels in activated T cells from SOCS3^{+/−} mice were 5–10 times lower than those in SOCS3^{+/+} T cells (Fig. 4 A), but the reduction did not alter T cell development based on cell surface markers (unpublished data). When T cells were stimulated with anti-TCR cross-linking, T cells from SOCS3 heterologous mice produced equivalent amount of IL-2 compared with that of SOCS3^{+/+} T cells (Fig. 4 B). In contrast, the CD28-mediated enhancement of IL-2 production was markedly augmented in T cells from SOCS3^{+/−} mice (Fig. 4 B). The IL-2 production levels in SOCS3^{+/−} T cells were consistently twofold higher than those of SOCS3^{+/+} T cells. These results clearly indicated that the decreased expression level of SOCS3 enhanced CD28-mediated IL-2 production ability in primary T cell activation.

SOCS3 Inhibited CD28-mediated IL-2 Promoter Activation. We further examined the molecular basis of the negative regulation of CD28 costimulation by SOCS3. To minimize the effect of endogenous CD28 molecules, mouse CD28 molecule was transfected into Jurkat cells. WT or mutated SOCS3 (R71E and F25A) was further cotransfected with the IL-2 promoter luciferase reporter gene, which contains CD28RE sequence. R71E is a substitution mutant at the SH2 domain whereas F25A contains a point mutation at the KIR. Both cannot inhibit IL-2–induced STAT5 activation (18 and unpublished data). The
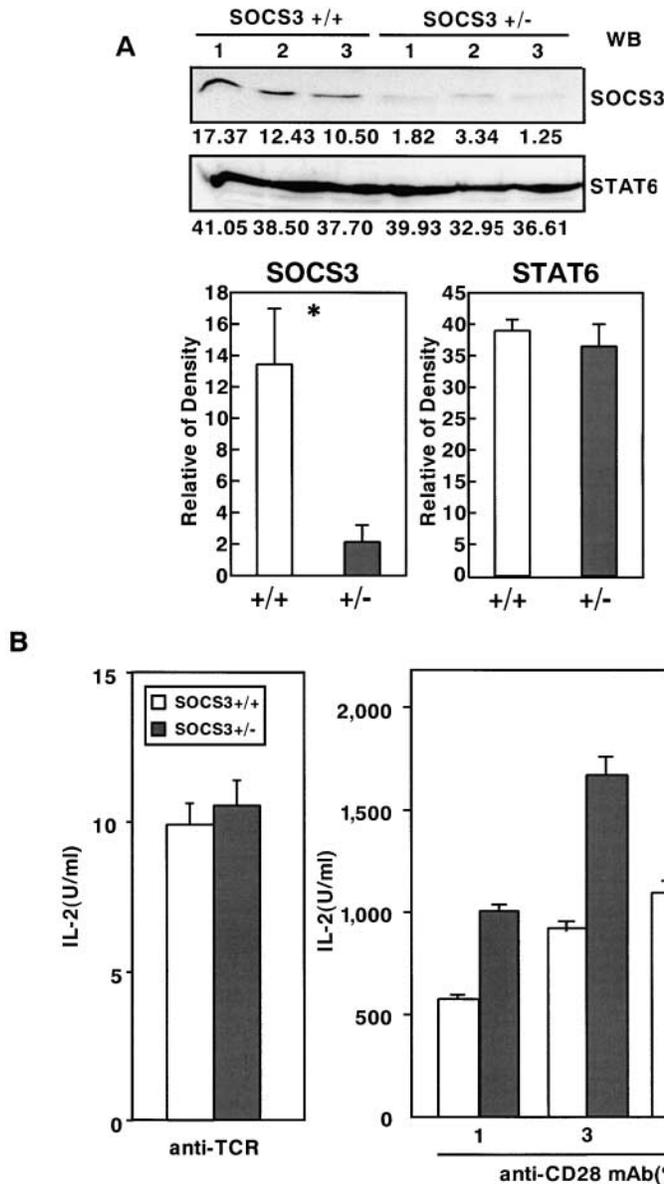
Figure 4. (A) SOCS3 protein expression in activated T cells from SOCS3^{+/+} and SOCS3^{+/−} mice. T cells from three SOCS3^{+/+} and SOCS3^{+/−} mice with C57BL/6 background was stimulated with anti-TCR and anti-CD28 mAb for 48 h. SOCS3 and STAT6 protein expression was analyzed by Western blotting using specific antibody. STAT6 expression is represented as an internal control. The mean ± SD of optimal densities was obtained from three independent experiments. *, P < 0.05 versus SOCS3^{+/+} mice. (B) T cells from three SOCS3^{+/+} and SOCS3^{+/−} mice with C57BL/6 background was stimulated with anti-TCR in the presence or absence of anti-CD28 mAb. Three different concentration of the culture supernatant of anti-CD28 mAb (1–10%) were added. The culture supernatants of the stimulated T cells were harvested at 48 h and IL-2 concentration was measured by ELISA. The mean ± SD shown was obtained from three mice.

Jurkat cells expressing mouse CD28 were stimulated with either anti–human CD3 plus anti–mouse CD28 mAb, PMA plus anti–mouse CD28 mAb, or PMA plus ionomycin. The overexpression of WT and F25A mutant inhibited CD28-mediated IL-2 promoter activation (Fig. 5, left and middle) but did not affect the CD28-independent IL-2 promoter activation using a combination of PMA and ionomycin (Fig. 5, right). On the other hand, the mutation at SH2 region (R71E) exhibited no inhibition for the IL-2 promoter (Fig. 5, middle). These results suggested that the SH2 domain is responsible for the inhibition of CD28-mediated IL-2 promoter activation and that SOCS3 possibly acts on CD28 costimulation through its SH2 domain.

Binding of SOCS3 to Tyrosine-phosphorylated CD28 Through Its SH2 Domain. Previous reports have clearly demonstrated that the inhibitory function of SOCS3 is based on the interaction with the tyrosine-phosphorylated JAK and/or the cytokine receptor (13, 18). Thus, we examined whether tyrosine phosphorylation of CD28 is critical for the interaction between CD28 and SOCS3. GST or GST fused to CD28 cytoplasmic domain (GST-CD28) were bacterially tyrosine phosphorylated and incubated with the cell extract from 293 cells expressing myc-tagged SOCS3. GST-CD28 proteins were precipitated by glutathione-Sepharose beads and coprecipitation of SOCS3 was assessed by blotting with anti-myc mAb. As shown in Fig. 6 A, SOCS3 specifically coprecipitated with the tyrosine-phosphorylated CD28 but not unphosphorylated CD28. To further determine the binding motif on SOCS3, CD28 was coexpressed in 293 cells with either WT or mutant SOCS3 and the association of SOCS3 was examined by precipitation with anti-CD28 mAb. WT and F25A were coprecipitated with the tyrosine-phosphorylated CD28 whereas R71E, an SH2 mutant, was not precipitated with CD28 (Fig. 6 B). To confirm this association in T cells from SOCS3 Tg mice, thymocyte extracts were immunoprecipitated with anti-CD28 mAb after activation

with anti-TCR and CD28 mAbs. In this experiment, F25A Tg mice were used to eliminate the alternative effect of inhibitory function for the cytokine signal. Consistently, T cells from F25A Tg mice showed an inducible association with CD28 when T cells were stimulated with anti-TCR plus CD28 mAb (Fig. 6 C), demonstrating that SH2 domain but not KIR on SOCS3 is critical for the association with the tyrosine-phosphorylated CD28.

Redundant Binding of SOCS3 to Tyrosine-phosphorylated CD28. It has been well documented that the tyrosine residue within the YMNM motif of the cytoplasmic region of CD28 is a target site for PI3-K and Grb2, which transduce signals downstream (24–28). We notice that the SOCS3 SH2 domain shows a sequence similarity to the PI3-K SH2 domain (unpublished data). Thus, it is reasonable to speculate that the expressed SOCS3 specifically binds to the YMNM motif on CD28 and inhibits the association of PI3-K to the phosphorylated CD28. To study this possibility, we attempt to compare the association of PI3-K to the phosphorylated CD28 between T cells from the littermate and Tg mice. Thymocytes from F25A Tg mice clearly revealed the reduction of PI3-K association to CD28 when cells were stimulated with the combination of anti-TCR and CD28 mAb (Fig. 6 C), suggesting that the forced expression of SOCS3 inhibited the association of PI3-K with CD28 in thymocytes. However, this inhibition was only observed in Tg-derived thymocytes but not in spleen T cells because the expression level of transgene in thymocytes was 10 times higher than that in spleen T cells (unpublished data).

We further studied whether SOCS3 specifically recognize the tyrosine residue (Y189) in the YMNM motif on CD28. Thus, a series of mutant CD28 were generated by either substitution at each tyrosine residue or deletion and were expressed in 293 cells with SOCS3 (Fig. 6 D). The tyrosine residue at 189 has been shown to be critical for the association with PI3-K and Grb family molecules (24). In-
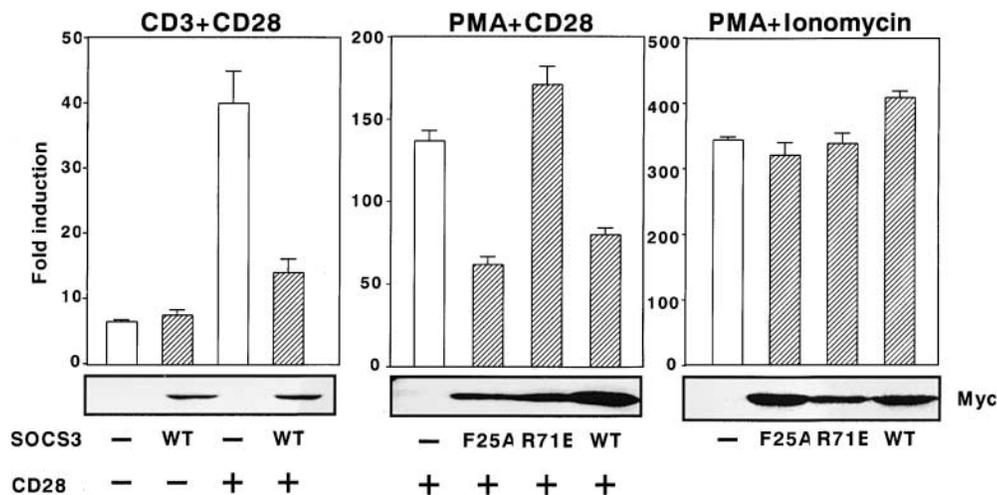
Figure 5. Inhibition of CD28-mediated IL-2 promoter activation by SOCS3. The IL-2 promoter (10 μg) and SOCS3 (5 μg) constructs were cotransfected into Jurkat cells and then cultured for 24 h. After transfection, cells were stimulated with anti-CD3 plus anti-CD28 mAb (top left), PMA plus anti-CD28 mAb (top middle), or PMA plus ionomycin (top right). WT, KIR mutant, and SH2 mutant of SOCS3 were represented as WT, F25A, and R71E, respectively. Protein expression of transfected WT and mutant SOCS3 was indicated by Western blotting with anti-Myc mAb (bottom columns). The data represents mean value of fold induction against the relative light unit of unstimulated Jurkat cells. The mean ± SD was obtained from three independent experiments.
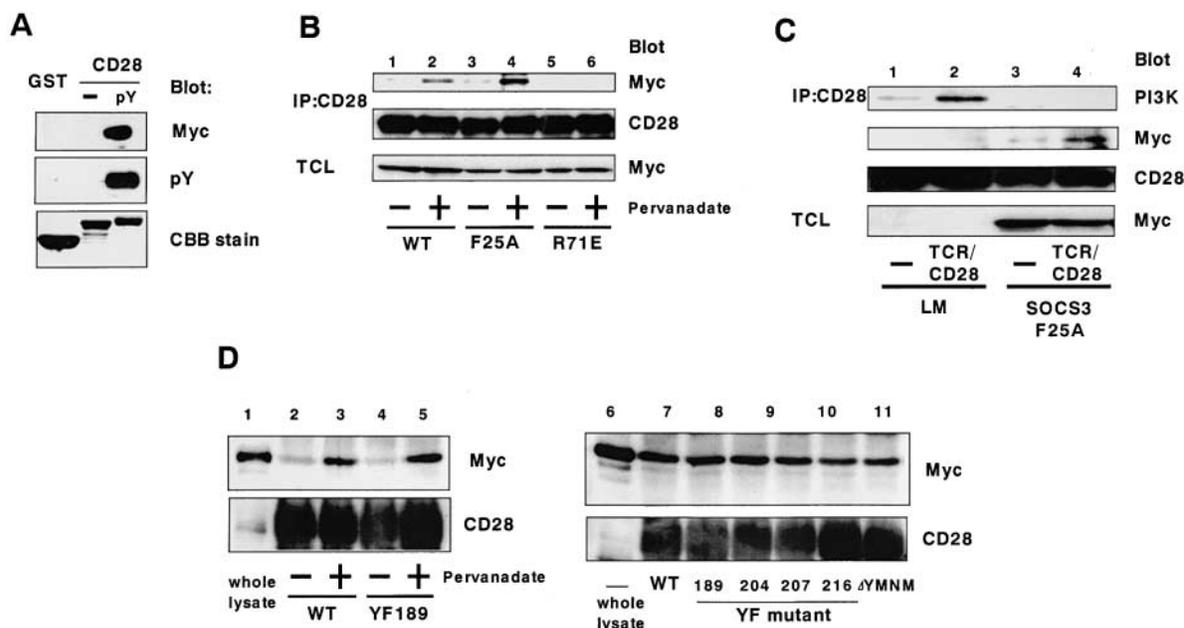
Figure 6. Binding SOCS3 to the phosphorylated CD28 molecule through the SH2 domain. (A) Binding specificity of SOCS3. Myc-SOCS3 was transiently expressed in HEK293 cells. The cell lysate was mixed with either GST-CD28 or GST-CTLA4 and the bound myc-SOCS3 was estimated by immunoblotting with anti-myc. Purified GST protein was detected by Coomassie Brilliant Blue stain. (B) Binding domain on SOCS3 to CD28. HEK293 cells cotransfected with the CD28 plasmid and either SOCS3, F25A, or R71E expression constructs. After 24 h, 293 cells were incubated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) pervanadate for 5 min. Cell extracts (1 mg) were immunoprecipitated by anti-CD28 antibody and immunoblotted with anti-myc or anti-CD28 antibodies. (C) Association of SOCS3 with CD28-reduced PI3-K binding. Thymocytes (10^8 cells) from littermate (LM; lanes 1 and 2) and F25A Tg (lanes 3 and 4) mice were stimulated with anti-TCR and anti-CD28 mAb for 2 min (lanes 2 and 4). The cell lysate was immunoprecipitated with anti-CD28 mAb and the association of PI3-K and SOCS3 was visualized by immunoblotting with either anti–PI3-K or anti-myc antibody. (D) Redundant association of SOCS3 with CD28. HEK293 cells cotransfected with WT SOCS3 and WT (lanes 1, 2, 3, and 7), YF mutants (lanes 4, 5, 8, 9, 10, and 11), or deletion mutant (lane 12) of CD28. After 24 h, 293 cells were incubated with (lanes 3, 5, and 6–12) or without (lanes 1, 2, and 4) pervanadate for 5 min. The immunoprecipitates with anti-CD28 antibody were immunoblotted with anti-myc or anti-CD28 antibodies.

deed, the substitution mutant, Y189F, abrogated binding to both PI3K or Grb2 and CD28-induced signaling was assessed by IL-2 production (unpublished data). However, when SOCS3 was transfected with 293 cells with either WT or Y189F CD28, SOCS3 was immunoprecipitated with WT CD28 as well as YF189 mutant (Fig. 6 D, lane 5). Unexpectedly, the association between CD28 and SOCS3 was not canceled in either of four substitution mutants (YF189, 204, 207, and 216) as well as the deletion of the YMNM motif of CD28 (Fig. 6 D, right). These result indicated that the association between CD28 and SOCS3 was dependent on the SH2 domain of SOCS3 and the tyrosine phosphorylation of CD28, however, multiple tyrosine phosphorylation sites of CD28 could interact with SOCS3. Nevertheless, the binding of SOCS3 protein to CD28 inhibited the association of PI3-K to tyrosine-phosphorylated CD28

As shown in Fig. 6 C, SOCS3 inhibited the association of PI3-K with CD28 when high amounts of SOCS3 protein were expressed. However, it is very difficult to conclude that the inhibition of the PI3-K association contributed to the SOCS3-mediated inhibition of CD28 costimulation in physiological conditions. Therefore, we next compared the association of PI3-K with the phosphorylated CD28 between DO11.10-derived Th1 and

Th2 cells on the basis of our previous finding that SOCS3 protein was exclusively expressed in Th2 but not Th1 cells (Fig. 7 A; references 36 and 37). PI3-K was coprecipitated with CD28 in Th1 cells after stimulation with anti-CD28 mAb. However, Th2 cells show a weak association between CD28 and PI3K even though the expression levels of CD28 and PI3K were indistinguishable between Th1 and Th2 cells (Fig. 7 A). These data suggest that inhibition of the association between CD28 and PI3-K by SOCS3 protein may occur at physiological levels. Furthermore, the CD28 dependency in cytokine production was different between Th1 and Th2 cells (Fig. 7 B). Th1 cytokines, such as IL-2 and IFN-γ, showed a greater fold induction mediated by CD28 costimulation than those of Th2 cytokines, IL-4 and IL-5 (Fig. 7 B). These results indicated that compared with Th1 cells, the effect of CD28 costimulation on cytokine production is decreased in Th2 cells that exclusively express SOCS3 protein.

Induction of SOCS3 Expression Reduced CD28-mediated IL-2 Production at Primary T Cell Activation. We have recently reported that SOCS3 was strongly and specifically expressed in the presence of IL-4 in naive T cells (unpublished data). Thus, we compared primary IL-2 production controlled by CD28 costimulation between the mice in the presence and absence of IL-4 receptor signaling. T cells
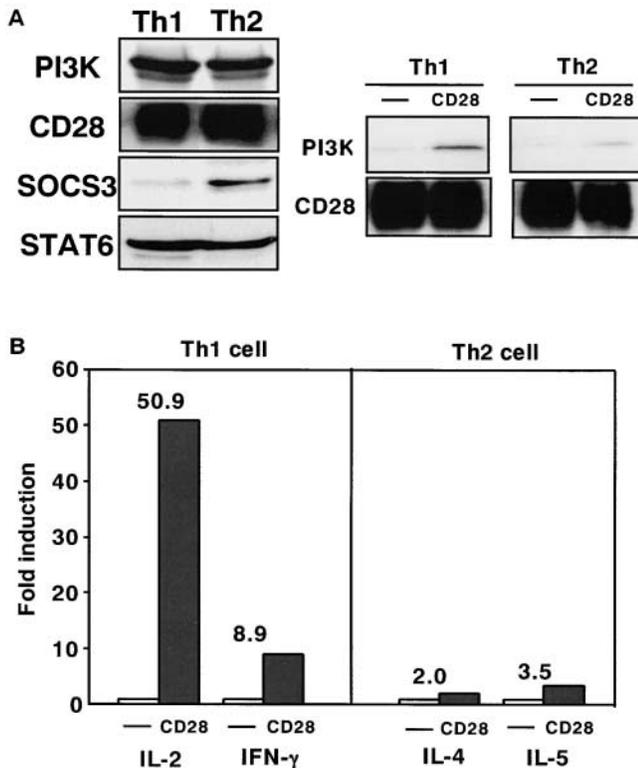
Figure 7. Expression of SOCS3 in Th2 cells impaired the association of PI3-K with CD28. (A) DO11.10 CD4+ T cells with BALB/c background were stimulated with OVA peptide under either Th1 or Th2 polarizing conditions. Protein expression of PI3-K, CD28, SOCS3, and STAT6 was analyzed 7 d after OVA stimulation (left). These Th1 and Th2 cells were stimulated with soluble anti-CD28 mAb (20 μg/ml) for 10 min and the cell extracts were precipitated with anti-CD28 mAb. The association of PI3-K was assessed by Western blot analysis with anti–PI3-K antibody (right). The same experiment was performed three times with the same result. (B) DO11.10-derived Th1 and Th2 cells were stimulated with immobilized anti-TCR mAb (10 μg/ml) in the presence or absence of anti-CD28 mAb (10% culture supernatant) and cytokine productions were measured. CD28-mediated cytokine productions are represented as fold induction versus the anti-TCR mAb-mediated cytokine production.

from DO11.10 and DO11.10 IL-4R$^{-/-}$ mice on BALB/c background were stimulated with OVA peptide in the presence of APCs and expression levels of IL-2 and SOCS3 were assessed after 48 h. The induction of SOCS3 expression was minimized in DO11.10 IL-4R$^{-/-}$ T cells (Fig. 8 A). However, DO11.10 IL-4R$^{-/-}$ T cells stimulated with BALB/c APCs produced twice as much IL-2 compared with that of DO11.10 T cells from IL-4R WT mice (Fig. 8 A). These data indicate that the effect of SOCS3 on IL-2 production was not only observed in anti-CD3 and CD28 antibody stimulation but also in the response to natural antigen and costimulators.

A similar relationship was observed between BALB/c and B10.D2 strains of mice that have a distinct ability in IL-4 production at primary T cell activation (38, 39). Naive CD4+ T cells from BALB/c mice secreted 10 times more IL-4 than that of B10.D2 mice (Fig. 8 B). Consistent

with the differential IL-4 production, the activated BALB/c T cells but not B10.D2 T cells showed clear SOCS3 expression. BALB/c T cells in which SOCS3 expression was induced by IL-4 had half the IL-2 production observed in B10.D2 T cells in response to CD3 and CD28 (Fig. 8 B). These results indicated that the IL-4–induced SOCS3 expression during primary T cell activation inhibited the IL-2 production after CD28 costimulation.

Discussion

Members of the SOCS family were discovered as negative regulators of cytokine signaling capable of inhibiting the JAK-STAT pathway (8–10, 40). Much evidence has indicated that SOCS3 can bind JAK tyrosine kinases and inhibit the JAK-STAT signaling activated by various cytokines including leukemia inhibitory factor, IL-2, IL-6, erythropoietin, and prolactin (7). This study describes that when SOCS3 is expressed in T cells, CD28-mediated IL-2 production is inhibited. The inhibitory action was distinct from that for cytokine signaling in which both the KIR and SH2 domain were indispensable whereas the SH2 domain was sufficient for the inhibition of CD28 costimulation. The expressed SOCS3 in early T cell activation may have an alternative role in prohibiting excessive T cell activation mediated by CD28 costimulation.

The sequence of SH2 domain on SOCS3 showed similarity to the PI3-K SH2 domain, suggesting the possibility that SOCS3 may share similar binding specificity with PI3-K. Therefore, we speculated a role of SOCS3 in the CD28 signaling pathway and thus studied CD28-mediated IL-2 production using Tg mice constitutively expressing SOCS3 protein and SOCS3$^{+/-}$ mice. Because SOCS3 expression levels were regulated by IL-4, we studied the relationship between SOCS3 expression and CD28-mediated IL-2 production in the conditions in which either the IL-4 signal or IL-4 was restricted (Fig. 8). The reduction of CD28-mediated IL-2 production was consistently observed along with the increased expression level of SOCS3 in all the systems examined in this study. These results clearly indicated that SOCS3 has an alternative function as a negative regulator for the CD28-mediated IL-2 production in primary T cell activation. SOCS3 mRNA was constitutively expressed in naive CD4 T cells and the expression was rapidly down-regulated by TCR cross-linking. However, the presence of CD28 costimulation could overcome SOCS3 expression within the first 48 h, suggesting that SOCS3 induced during early T cell activation may account for negatively controlling the excessive progression of CD28-mediated IL-2 production.

SOCS3 not only play a role during primary T cell activation but may also be important in committed Th cells. It has been recently reported that Th2 cells exclusively expressed SOCS3 protein (Fig. 6; references 36 and 37). The differential expression of SOCS3 strongly correlated with the dependency for CD28 costimulation during cytokine production from DO11.10 Tg-derived Th1 and Th2 cells. Th1 cytokines, such as IL-2 and IFN-γ, showed prominent
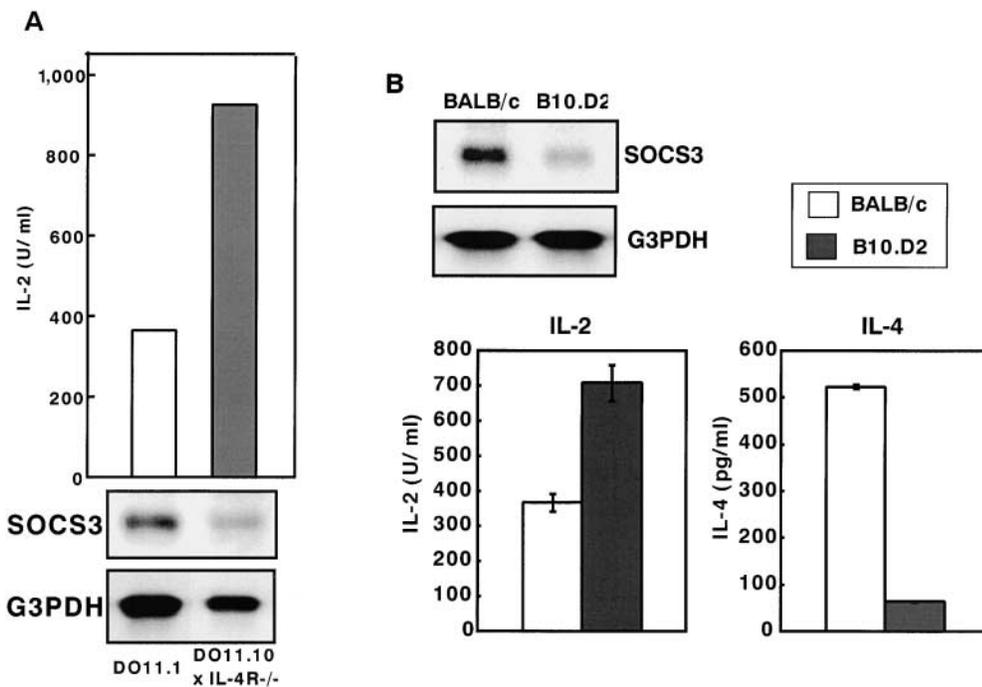
Figure 8. IL-4–induced SOCS3 expression negatively regulated CD28-mediated IL-2 production. (A) CD4+ T cells were prepared from DO11.10 or DO11.10 IL-4R–deficient mice with BALB/c background and stimulated with OVA peptide in the presence of BALB/c APC and IL-4 for 48 h. SOCS3 and G3PDH mRNA expression (bottom) and IL-2 production (top) were analyzed. The same experiment was performed three times with the same result. (B) CD4+ T cells were prepared from BALB/c and B10.D2 strains of mouse. The cells were stimulated with anti-TCR and anti-CD28 mAb in unskewed condition for 48 h. SOCS3 and G3PDH mRNA expression and IL-2 and IL-4 production were analyzed. The mean ± SD in IL-2 and IL-4 production shown was obtained from three mice.

CD28-mediated enhancement whereas a large amount of the Th2 cytokines, IL-4 and IL-5, were capable of secreting with TCR cross-linking, and the dependency for CD28 costimulation was less than that of the Th1 cytokines (Fig. 7 B). These results were consistent with the observation that the presence of SOCS3 attenuated CD28-mediated IL-2 production in early T cell activation. Interestingly, Th2 cells exhibited very low association of PI3-K with CD28 when cells were activated with anti-CD28 mAb. Despite this, Th1 cells showed a clear association between PI3-K and CD28 (Fig. 7 A), suggesting that SOCS3 inhibits CD28-dependent signaling through the reduction of the association of PI3-K with CD28.

Our biochemical characterization and in vitro mutational analysis have demonstrated that the mechanism of an SOCS3 inhibition of the CD28 costimulation is distinct from the inhibition of cytokine signaling as in the latter interactions at both the SH2 domain and KIR on SOCS3 are required. Our data show that the SH2 domain is sufficient with SOCS3 interaction with the phosphorylated CD28 (Fig. 6). Previous reports have provided substantial evidence that the tyrosine residues within the CD28 YMNM motif are critical for binding Grb2 and the PI3-K p85 subunit by which the CD28-mediated signal is transduced downstream (24–28). Therefore, we speculated that the YMNM motif on the cytoplasmic region of CD28 would be a target site for SOCS3 binding. The low association of PI3-K with CD28 that we observed in SOCS3 Tg-derived thymocytes (Fig. 6 C) and Th2 cells (Fig. 7 A) supports our speculation. However, SOCS3 can redundantly bind to all four phosphorylated tyrosine residues on CD28 (Fig. 6 D), indicating that the YMNM motif is not a specific target of SOCS3. Therefore, although the reciprocal interaction of

CD28 with PI3-K and SOCS3 partly involves the inhibitory function for CD28-mediated IL-2 production, it remains a possibility that a different mechanism was responsible for the inhibition of CD28 costimulation by SOCS3. Additional investigation is necessary to elucidate the inhibitory mechanisms of CD28-mediated responses by SOCS3.

The activation and subsequent proliferation of peripheral T cells requires the engagement of the TCR. The members of the SOCS family have been well documented as negative regulators of cytokine signaling via inhibition of the JAK-STAT pathway. However, there has been little evidence that they have broader functions across T cell and cytokine receptor signaling pathways. A recent report has described that CIS-1 play a role in enhancing mitogen-activated protein kinase activation initiated by TCR stimulation. The CIS-1–increased MAP kinase was elucidated with a direct interaction of CIS-1 and protein kinase Cθ (41). In biochemical characterization, SOCS3 has been shown to associate with T cell–specific tyrosine kinase p56lck in in vitro binding assays (8). Recently, it has been reported that SOCS3 can associate with a catalytic subunit of calcineurin, resulting in the inhibition of calcineurin-dependent NF-ATp activation (33). However, either enhanced or reduced expression of SOCS3 did not have marked effects on T cell development nor TCR-mediated IL-2 production (Fig. 1). Consistently, SOCS3-deficient mice that the T cells reconstituted in JAK3-deficient mice with SOCS3-deficient fetal liver cells showed no alteration in T cell development nor the IL-2–induced proliferative response (22). T cells from SOCS3 Tg and heterologous mouse exhibit reciprocal alteration of the CD28-mediated IL-2 production in primary T cell activation. Similarly, T cells from CD28-deficient mice impaired in the primary T

cell activation mediated by the B7–CD28 interaction but not T cell development nor TCR-mediated IL-2 production (42). Thus, it is reasonable to speculate that a functional target in TCR-related signaling is the CD28-mediated signaling. The rapidly induced SOCS3 during the early T cell activation may play an important role to negatively regulate the excessive primary IL-2 production induced by CD28 costimulation.

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