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Loss of Suppressor of Cytokine Signaling 1 in Helper T Cells Leads to Defective Th17 Differentiation by Enhancing Antagonistic Effects of IFN-γ on STAT3 and Smads

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Suppressor of cytokine signaling 1 (SOCS1) is an important negative regulator for cytokines; however, the role of SOCS1 in Th17 differentiation has not been clarified. We generated T cell-specific SOCS1-deficient mice and found that these mice were extremely resistant to a Th17-dependent autoimmune disease model, experimental autoimmune encephalomyelitis. SOCS1-deficient naive CD4+ T cells were predominantly differentiated into Th1 and poorly into Th17 in vitro. These phenotypes were canceled in IFN-γ−/− background, suggesting that a large amount of IFN-γ in SOCS1-deficient T cells suppressed Th17 differentiation. IL-6 plus TGF-β enhanced retinoic acid receptor-related orphan receptor (ROR)γt expression and suppressed IFN-γ production in wild-type T cells, whereas these effects were severely impaired in SOCS1-deficient T cells. These phenotypes can be partly explained by STAT3 suppression by enhanced SOCS3 induction through hyper-STAT1 activation in SOCS1-deficient T cells. In addition, SOCS1-deficient T cells were much less sensitive to TGF-β. Suppression of Th1 differentiation by TGF-β was impaired in SOCS1-deficient T cells. TGF-β-mediated Suppressor of Cytokine Signaling 1 transcriptional activity was severely inhibited in SOCS1-deficient cells in the presence of IFN-γ. Such impairment of TGF-β functions were not observed in SOCS3-overexpressed cells, indicating that suppression of Smads was independent of SOCS3. Therefore, SOCS1 is necessary for Th17 differentiation by suppressing antagonistic effect of IFN-γ on both STAT3 and Smads. Induction of SOCS3 can partly explain IFN-γ-mediated STAT3 suppression, while other mechanism(s) will be involved in IFN-γ-mediated Suppressor of Cytokine Signaling 1 suppression. SOCS1-deficient T cells will be very useful to investigate the molecular mechanism for the STAT1-mediated suppression of Th17 development. The Journal of Immunology, 2008, 180: 3746–3756.

Upon activation, T cells undergo distinct developmental pathways, retaining specialized properties and effector functions. Th cells are traditionally thought to differentiate into Th1 and Th2 cell subsets (1). Th1 polarization is primarily driven by IL-12 and IFN-γ, while Th2 polarization is primarily driven by IL-4. These respective cytokines signal via STAT4, STAT1, and STAT6 to directly control the transcription factors T-bet and GATA3, which, in turn, determine Th1 and Th2 differentiation, respectively (2). Th1 cells produce IFN-γ, which facilitates their differentiation while inhibiting IL-4-mediated Th2 differentiation. Reciprocally, Th2 cells produce IL-4 and IL-10, which strongly inhibits IL-12/IFN-γ-driven Th1 differentiation. Recently, it has been described that a novel helper T cell subset, Th17, produces IL-17 and is distinct from Th1 or Th2 cells (3–7). The Th17 differentiation of naive T cells is initiated by IL-6 and TGF-β (8–10). In addition, IL-23 as well as IL-21 is thought to be a key cytokine for the maturation and/or maintenance of Th17 cells (11–13). All of IL-6, IL-21, and IL-23 activate STAT3 and STAT3 is thought to be essential for Th17 differentiation (12–16). It has also been reported that STAT3 plays a critical role in the induction of the retinoic acid receptor-related orphan receptor γt (RORγt),3 which directs Th17 cell differentiation by inducing the IL-23R (17). RORγt could be a STAT3 target analogous to T-bet for STAT4 and to GATA3 for STAT6. However, the role of TGF-β in Th17 has not been clarified.

In addition to a promoting role of STAT3 in Th17 differentiation, STAT1 and STAT4 have been implicated in negative roles at the early stage of Th17 differentiation (6, 7). STAT1 activated by IFN-γ and IL-27 also suppressed Th17 differentiation (18, 19). However, IL-6, IL-23, and IL-27 all activated both STAT1 and

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3 Abbreviations used in this paper: RORγt, retinoic acid receptor-related orphan receptor γt; SOCS, suppressor of cytokine signaling; cKO, conditioned knockout; Ct, control; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; WT, wild type; m, mouse; h, human; Tg, transgenic; MEF, mouse embryonic cell.
that SOCS1 is critical in Th17 differentiation by maintaining IFN-γ enhancement of IL-17 production induced by IL-23 or IL-6 plus SOCS1.

The suppressors of cytokine signaling (SOCS) and cytokine-inducible SH2 protein family are a family of intracellular proteins, several of which have been shown to regulate the responses of immune cells to cytokines (22, 23). The discovery of the SOCS proteins apparently defined an important mechanism for the negative regulation of the cytokine-JAK-STAT pathway; however, recent studies using gene-disrupted mice revealed unexpected profound roles of SOCS proteins in many immunological processes (22). SOCS1 is a potent inhibitor of signaling events stimulated by both IFN-γ and IL-4 since, in the absence of the SOCS1 protein, both STAT1 and STAT6 are highly activated and, subsequently, T cells are unconditionally hyperactivated (22, 24). In contrast, SOCS3 has been implicated mostly in the regulation of STAT3 activated by IL-6, G-CSF, and leptin (25–27). We have shown that mice lacking SOCS3 in T cells showed reduced Th1 and Th2 responses and that SOCS3-deficient CD4+ T cells produced more TGF-β, suggesting that SOCS3-deficient T cells preferentially differentiate into Th3 cells which produce higher levels of TGF-β (28). However, paradoxically, SOCS3-deficient T cells showed a clear enhancement of IL-17 production induced by IL-23 or IL-6 plus TGF-β (16). STAT3 is hyperactivated in SOCS3-deficient T cells in response to IL-23 or IL-6 (16, 28). Thus, SOCS3 also plays a suppressive role in Th17 induction by negatively regulating STAT3 activation.

In this study, we generated T cell-specific SOCS1-deficient knockout (cKO) mice and found that SOCS1-deficient (SOCS1del/del) CD4+ T cells predominantly differentiated into Th1 cells and poorly into Th17 cells. This phenotype was largely dependent on IFN-γ. We found that hyperinduction of SOCS3 by the SOCS1 deficiency is one of the mechanisms of reduced Th17 differentiation. In addition, we found that TGF-β signaling was severely impaired in IFN-γ-treated SOCS1-deficient cells. We concluded that SOCS1 is critical in Th17 differentiation by maintaining proper STAT3 and Smad transcriptional activities.

Materials and Methods

**Generation of conditional SOCS1-deficient mice**

Conditional targeting was done as previously described (27). To construct the targeting vector, we cloned an XhoI-EcoRI fragment of 1.3 kb containing a whole SOCS1-coding sequence into the BamHI site of the pflox vector. An AccI-XhoI fragment of 5.8 kb and an EcoRI-KpnI fragment of 1.3 kb were inserted adjacent to the coding region. The targeting construct was linearized and transfected into the J1 embryonic stem cells by electroporation. Single-cell ES clones were cultured in Iscoves modified Dulbecco’s medium, and selected with ganciclovir as described. We identified deletion inserts by Southern blot analysis. Lck-cre-transgenic (Tg) mice (C57BL/6 background) were bred with SOCS1flox/flox mice (129Sv/6 background) to generate mice in which SOCS1flox/flox; control) were used for intercrossing and further analyses. T cells from SOCS1 cKO mice were designated as SOCS1lac/del T cells. Littermate controls (SOCS1flox/flox; control) were used for all experiments. Mice were kept in specific pathogen-free facilities in the Biomedical Research Station Animal Facility of Kyushu University (Fukuoka, Japan). All experiments using these mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Kyushu University.

**Other mice**

IFN-γ−/− and IFN-γ−/−/SOCS1−/− mice with the C57BL/6 background and SOCS3 Tg mice were described previously (29, 30).

**Experimental autoimmune encephalomyelitis (EAE) induction and disease scoring**

For the induction of EAE, mice were immunized s.c. on day 0 with 100 μg/mouse myelin oligodendrocyte glycoprotein (MOG) peptide (aa 35–55) (MEGVWYRSPSPSRVYHLRNGK) (Bexis, emulsified in CFA (supplemented with 5 ng/ml Mycobacterium tuberculosi)), and injected i.p. on days 0 and 2 with 500 ng/mouse of pertussis toxin (Calbiochem) as described previously (31). The following clinical scoring system was used: 0, no disease; 1, tail limpness; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness; 5, quadriplegia; and 6, death. The scoring was done every 24 h until day 30.

Adoptive transfer of T cells from WT and SOCS1 cKO mice was performed as described previously (32). In brief, mice were immunized with MOG peptide as described above and, after 10 days, draining lymph nodes were harvested for isolation of CD4+ T lymphocytes. The cells were cultured with MOG peptide (0.2 ng/ml) and IL-12 (10 ng/ml) in the presence of splenocytes for 7 days, then 1 × 106 cells were transferred into WT mice as well as with 500 ng of pertussis toxin (both immediately and 2 days after cell transfer). The animals were observed daily and neurological effects were quantified as described above.

**Isolation of CD4+CD25+ T cells**

Single-cell suspensions from the spleen and lymph nodes were first negatively depleted by staining with anti-B220-biotin, anti-CD11b-biotin, anti-Ter119-biotin, anti-DX5-biotin, and anti-CD8a-biotin (eBioscience) at a 1/125 dilution for 20 min on ice, followed by incubation with anti-biotin magnetic microbeads (Miltenyi Biotec) to a 1/20 dilution for 20 min on ice. The depleted fraction was stained with anti-CD4- FITC and anti-CD25-PE. Cell sorting was performed by FACSAria (BD Biosciences) to obtain a pure population of primary CD4+ CD25+ T cells (>98% purity).

**In vitro T cell differentiation assay**

CD4+CD25− T cells (3 × 105/well) were cultured at 37°C (5% CO2) in RPMI 1640 (Invitrogen Life Technologies) containing 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-μg/ml plate-bound anti-TCR mAb (clone 145-2C11) and 0.5 μg/ml soluble anti-CD28 mAb (eBioscience); 40 U/ml mouse (m) IL-2 (mIL-2; R&D Systems) supplemented with 1 μg/ml anti-IFN-γ and 1 μg/ml anti-IL-4 Abs for Th0 differentiation, 10 ng/ml mIL-17p70 (R&D Systems) and 1 μg/ml anti-IL-4 Ab for Th1 differentiation, 10 ng/ml mIL-4 (PeproTech) and 10 ng/ml anti-IL-12 Ab (IL-12p40 homodimer; R&D systems) for Th2 differentiation, and 1 μg/ml human TGF-β1 (hTGF-β1; R&D Systems) and 10 ng/ml human (h) IL-6 (R&D Systems) for Th17 differentiation. Cells were collected after 5 days, washed, and used for experiments.

**Surface and intracellular cytokine staining**

For intracellular cytokine staining, cultured cells were restimulated for 8 h with 50 nM PMA (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich) and 1 μM brefeldin A (eBioscience). Surface staining was performed for 15 min with the corresponding mixture of fluorescently labeled Abs. After surface staining, the cells were suspended in Fixation Buffer (eBioscience), and intracellular cytokine staining was performed as per the manufacturer’s protocol using anti-IFN-γ-FITC, anti-IL-4-PE (eBioscience), and anti-IL-17-PE (BD Pharmingen).

**FACS analysis**

FACS analysis was performed on a FACS Calibur (BD Biosciences) instrument and analyzed using FlowJo software (Tree Star).

**ELISA**

Cytokine ELISAs were performed using the appropriately matched Abs (eBioscience) as previously described (28). OD at 450 nm was read on a Labsystems Multiscan MS.

**Ex vivo T cell responses**

Single-cell suspensions from the spleen and draining lymph nodes were prepared 15 days after immunization. CD4+ T lymphocytes were purified by anti-CD4 magnetic microbeads (Miltenyi Biotec) and MACS columns (purity was >95%). CD4+ T cells (2 × 105/well) were cultured in 96-well
flat plates with $2 \times 10^4$ irradiated splenocytes in the presence of 0.2 μg/ml anti-TCR mAb (clone 145-2C.11) or 20 μM MOG peptide. Cultures were performed at 37°C (5% CO$_2$) for 60 h and pulsed with 1 μCi of [H]thymidine (1 μCi/ml; Amersham Biosciences) during the final 12 h of incubation before harvesting. [H]Thymidine incorporation was determined on a microplate scintillation counter (PerkinElmer). Supernatants were collected at 48 h and IFN-γ and IL-17 were measured by ELISA (eBioscience).

**RT-PCR and real-time RT-PCR**

Total RNA was extracted from primary or cultured CD4$^+$ T cells with RNA ISO (Takara) according to the manufacturer’s instructions. cDNA was performed using the one-step RT-PCR kit (Applied Biosystems) according to the manufacturer’s instructions. The following oligonucleotides were used for PCR: 5’-ACCCACCTGTGCTGTA-3’ and 5’-CACGCAAGCAGAACTGGTTCTTC-3’; 5’-TCAGGT-AGTCACGGAGTACC-3’ and 5’-GGTGTCCTTTCC-3’ (ROKR); 5’-GCACAGGGACTGCTTCTTC-3’ and 5’-TCAGGT-AGTCAAGAGCTGTG-3’ (SOCS1); 5’-GTTGACGCGTCAAGAGCTGTG-3’ (SOCS3); 5’-TCGTTGACAGTGGTG-3’ and 5’-TTCCAGGTGCATGTCAT-3’ (IL-17A); 5’-AACAGGTCCTGTATCCCTCTCC-3’ and 5’-GCCCGTTGGAAGAGGAGGAGG-3’ (SOCS3); 5’-GTTGACGCGTCAAGAGCTGTG-3’ and 5’-TTCCAGGTGCATGTCAT-3’ (IL-17A); 5’-AACAGGTCCTGTATCCCTCTCC-3’ and 5’-GCCCGTTGGAAGAGGAGGAGG-3’ (SOCS3); and 5’-GTTGACGCGTCAAGAGCTGTG-3’ and 5’-TTCCAGGTGCATGTCAT-3’ (IL-17A).

Real-time RT-PCR was described previously (33). The SYBR Green PCR Master Mix (Applied Biosystems) was used for the detection of the target genes. The comparative threshold cycle method and an internal control (G3PDH) were used to normalize the expression of the target genes.

**Western blot analysis**

Phosphorylation was analyzed by SDS-PAGE and Western blotting as described previously (27, 28), with the following Abs: anti-phospho-STAT1 (Tyr701; pY-STAT1), anti-phospho-STAT3 (Tyr705; pY-STAT3; Cell Signaling Technology) anti-STAT1 (E23), anti-STAT3 (C20), anti-ERK2 (C14; Santa Cruz Biotechnology), anti-Smad2 (BD Pharmingen), and anti-SOCS3 (C204; IBL). The blot was visualized with the appropriate HRP-conjugated secondary Abs using the Super Signal West Pico Chemiluminescent Substrate (Pierce) or Immobilon Western Chemiluminescent HRP Substrate (Millipore).

**Reporter assay and electroporation**

The Smad-binding element (SBE) luciferase reporter plasmid was previously described (34). Reporter gene assays were performed as described previously (31, 35). SOCS1-deficient and STAT1-deficient mouse embryonic fibroblast (MEF) cells were prepared as described elsewhere (36). For the reporter gene assay in primary T cells, plasmids were introduced into CD4$^+$ CD25$^-$ T cells using an Amaxa Mouse T Cell Nucleofactor Kit (Amansa Biosystems) according to the manufacturer’s protocol. After electroporation, T cells were activated with 1 μg/ml plate-bound anti-TCR mAb and 0.5 μg/ml soluble anti-CD28 mAb with or without 2 ng/ml hTGF-β1 and 100 U/ml mIFN-γ for 8 h. Then cells were lysed and the luciferase activity was measured.

**Statistical analysis**

For statistical analysis, all $p$ values were calculated with the one-tailed Student t test.

**Results**

Generation of SOCS1 cKO mice

To delete the SOCS1 gene in a T cell-specific manner, first, mice homozygous for the loxP-flanked (floxed) SOCS1floxed/flox genes were generated. A targeting vector was constructed so that the entire SOCS1-coding region could be deleted by the expression of Cre protein (Fig. 1A). SOCS1floxed/flox (SOCS1floxed/flox) mice were then crossed with proximal p56lck promoter-cre (Lck-Cre) Tg mice as described previously (28). Resulting SOCS1floxed/flox,....
Lck-Cre Tg mice were designated as SOCS1 cKO mice. SOCS1 wild-type (WT) alleles, floxed alleles, and the Lck-Cre transgene were determined by PCR using genomic DNA from tails and T cells. Deletion of the SOCS1 flox gene was specific to T cells and no deletion was observed in B cells, dendritic cells, or other major tissues in cKO mice (Fig. 1, B and C).

As reported (37), most SOCS1 cKO mice appeared normal and fertile until the age of 4 mo (Fig. 1D). In contrast, SOCS1 flox/flox; Tie2-Cre Tg mice (Tie2-2-CKO), in which the Cre gene was expressed in all hemopoietic cells (25), died within 50 days after birth of diseases very similar to those of SOCS1−/− mice (Fig. 1D). These data confirmed that SOCS1 deficiency in not only T cells but also APCs are required for acute fatal inflammatory diseases found in SOCS1−/− mice (38). Consistent with a previous report (37), the populations of CD4 single-positive T cells in the thymus and spleen were reduced, whereas that of CD8 single-positive T cells was increased (data not shown). Few immunological diseases were spontaneously developed within 3 mo after birth.

**Reduced EAE in SOCS1 cKO mice**

SOCS1 has been shown to be important for selection in the thymus and for peripheral tolerance (22, 23, 38, 39). To define the function of SOCS1 in autoimmune diseases, we performed the EAE model which is induced by MOG peptide/CFA immunization. Unexpectedly, T cell-specific SOCS1 cKO mice were extremely more resistant to EAE than SOCS1 flox/flox (Ct) littermates (Fig. 2A). To confirm that SOCS1-deficient CD4+ T cells are responsible for such reduced EAE in SOCS1 cKO mice, we examined a secondary transfer EAE model by adoptively transferred MOG-specific CD4+ T cells from WT or SOCS1-cKO mice into WT mice. Mice that received CD4+ T cells developed severer EAE than those that received SOCS1del/del CD4+ T cells (Fig. 2B). This indicates that SOCS1-deficient CD4+ T cells possessed lower potency to develop EAE than WT T cells. To determine the mechanism of amelioration of EAE by SOCS1 deletion in T cells, we examined helper T cell differentiation, since EAE is shown to be tightly dependent on Th17 cells (5, 8, 9). Fifteen days after EAE induction, T cells from control and cKO mice were restimulated with MOG peptide or anti-TCR Ab, after which proliferation and cytokine production were examined. As shown in Fig. 2, C and D, proliferation of CD4+ T cells from control and cKO mice was similar. Although the levels of IFN-γ were higher in T cells from cKO mice than in T cells from control mice, IL-17 levels were much lower in T cells from cKO mice stimulated with anti-TCR Ab or Ag peptide. IL-4 was below detection levels in both control and cKO mice in our immunizing conditions. Thus, reduced EAE in cKO mice may be due to reduced Th17 differentiation and enhanced Th1 differentiation.

**Th1 hyperpolarization and reduced Th17 differentiation in SOCS1-deficient T cells in vitro**

To further investigate Th17 differentiation in SOCS1 del/del CD4+ T cells, naive CD4+ T cells were differentiated in vitro (Fig. 3). By FACS and ELISA, strong induction of IFN-γ-producing cells was observed in SOCS1 del/del CD4+ T cells under the Th0 differentiation condition (anti-TCR Ab, anti-IL-4, and anti-IFN-γ Abs; Fig. 3A) and even under the Th2 differentiation condition (anti-TCR Ab, IL-4, and anti-IL-12 Abs). We confirmed that a combination of IL-6 and TGF-β with the anti-TCR Ab (the Th17 differentiation condition) resulted in the robust induction of IL-17 but very low levels of IFN-γ in control (SOCS1 flox/flox) cells (Fig. 3B, left). In contrast, SOCS1 del/del CD4+ T cells displayed a marked reduction in IL-17-producing cells and a strong induction of IFN-γ-producing cells under the Th17 differentiation condition (Fig. 3B, left). We confirmed hyper-Th1 and reduced Th17 induction in SOCS1 del/del T cells by ELISA for IL-17 and IFN-γ (Fig. 3B, right).

Reduced Th17 differentiation was also confirmed by Th17-specific gene expression (Fig. 3C). IL-17A, RORγt, and IL-23R expressions were enhanced in control T cells under the Th17 differentiation condition, whereas it was barely observed in SOCS1 del/del T cells. Th17 has also been shown to be induced by IL-23 in the presence of anti-IFN-γ and anti-IL-4 Abs (6, 7). Consistent with reduced IL-23R expression, IL-17 production was also very low in SOCS1 del/del T cells under an IL-23-mediated Th17 differentiation condition (Fig. 3D). To confirm that
SOCS1 plays an essential role in the IL-17/IFN-γ production in T cells, we restored the SOCS1 gene into SOCS1−/− T cells by crossing SOCS1−/− mice with SOCS1 Tg mice (38). Overexpression of the SOCS1 gene in SOCS1−/− T cells (designated as SOCS1+/−-Tg) resulted in restored IL-17 production and reduced IFN-γ production (Fig. 3E). Reduced IFN-γ production is probably because exogenous SOCS1 levels by the transgene were higher than endogenous SOCS1 levels (38). SOCS1 probably preferentially suppresses IFN-γ, whereas it does not suppress IL-6 signaling as efficiently compared with IFN-γ (23).
FIGURE 4. IFN-γ-dependent suppression of differentiation by SOCS1 deficiency. A and B, Cytokine profiles were determined by intracellular staining and ELISA. Naïve CD4+CD25+ T cells from indicated mice were activated in vitro under the Th17 differentiation condition (1 ng/ml hTGF-β1 and 10 ng/ml hIL-6) in the presence or absence of exogenous mIFN-γ (10 U/ml). Cytokine levels were determined by FACS (A) and ELISA (B) after restimulation (as described in Fig. 3). One representative data of two independent experiments are shown. Error bars represent SD. C, RT-PCR (left panel) and real-time RT-PCR (right panel) of IFN-γRs for Th17 cells. Total RNA was extracted from C5 or SOCS1del/del CD4+ T cells activated in vitro for 5 days under the Th17 differentiation condition.

These data indicate that SOCS1 levels in T cells regulate the Th1/Th17 balance.

Reduced Th17 differentiation of SOCS1-deficient T cells was IFN-γ dependent

It has been shown that the IFN-γ/STAT1 pathway inhibits Th17 differentiation (6, 7, 18, 19). We hypothesized that Th17 development was reduced in cKO mice because SOCS1del/del T cells polarized into Th1 and produced very high levels of IFN-γ. To test this hypothesis, we compared Th17 differentiation in vitro. IL-17 production was approximately two times higher in IFN-γ−/− T cells than in WT T cells as determined by intracellular cytokine staining and ELISA (Fig. 4, A and B). IL-17 production was similar between IFN-γ−/− and IFN-γ−/− SOCS1−/− T cells (Fig. 4, A and B), indicating that suppression of Th17 differentiation in SOCS1-deficient T cells was mostly dependent on IFN-γ.

To elucidate the role of IFN-γ in Th17 differentiation in SOCS1-deficient T cells, we examined Th17 differentiation in the presence of exogenous IFN-γ in vitro. Treatment of WT T cells with exogenous IFN-γ only slightly reduced IL-17 production and Th17 differentiation, while an ~25–50% reduction of Th17 cells was observed in IFN-γ−/− T cells (Fig. 4, A and B). These were confirmed by ELISA (Fig. 4B). These data suggest that endogenous IFN-γ substantially suppressed Th17 in WT T cells. In contrast, exogenous IFN-γ suppressed the Th17 differentiation of IFN-γ−/− SOCS1−/− T cells much more profoundly (up to 80%) than that of IFN-γ−/− T cells (Fig. 4, A and B). Since there was no significant difference in the IFN-γR expression levels between WT and SOCS1del/del Th17 cells (Fig. 4C), it was probably due to stronger IFN-γ signaling in SOCS1-deficient T cells. Thus, SOCS1 is necessary for protecting CD4+ T cells from the strong effect of IFN-γ on Th17 repression.

Enhanced STAT1 activation and reduced STAT3 activation in SOCS1-deficient T cells

The molecular mechanism for the suppression of Th17 differentiation by IFN-γ has not been established; therefore, SOCS1-deficient T cells could be good tools to investigate this mechanism. Thus, we examined the effect of SOCS1 deficiency in cytokine signaling in the Th17 differentiation condition. As shown in Fig.
5A, IL-6 plus TGF-β stimulation resulted in the transient phosphorylation of STAT1 and the sustained phosphorylation of STAT3 in control (SOCS1<sup>fl/fl</sup>) primary CD4<sup>+</sup> T cells. Whereas in SOCS1<sup>del/del</sup> CD4<sup>+</sup> T cells, STAT1 activation was sustained at 1 and 3 h and STAT3 activation was strongly suppressed after stimulation with IL-6 plus TGF-β (Fig. 5A). Then we compared STAT1 and STAT3 activation among IFN-γ<sup>-/-</sup> (WT), IFN-γ<sup>-/-</sup>, and IFN-γ<sup>-/-</sup>SOCS1<sup>-/-</sup> T cells. As shown in Fig. 5B, STAT1 activation was similar among WT, IFN-γ<sup>-/-</sup>, and IFN-γ<sup>-/-</sup>SOCS1<sup>-/-</sup> T cells. However, STAT3 phosphorylation was much higher in IFN-γ<sup>-/-</sup> and IFN-γ<sup>-/-</sup>SOCS1<sup>-/-</sup> T cells than in WT T cells, and it was similar between IFN-γ<sup>-/-</sup> and IFN-γ<sup>-/-</sup>SOCS1<sup>-/-</sup> T cells (Fig. 5B). Therefore, reduced STAT3 activation in SOCS1<sup>del/del</sup> T cells could contribute to reduction in Th17 differentiation, while higher STAT3 activation in both IFN-γ<sup>-/-</sup> and IFN-γ<sup>-/-</sup>SOCS1<sup>-/-</sup> T cells probably resulted in enhanced Th17 differentiation.

SOCS3 induction is a cause of the reduction in STAT3 activation

To further investigate the mechanism for reduced STAT3 phosphorylation in SOCS1-deficient T cells, we examined the levels of SOCS3, a strong inhibitor of IL-6 signaling. SOCS3 levels were compared among naive WT (Ct), SOCS1<sup>del/del</sup>, IFN-γ<sup>-/-</sup>, and IFN-γ<sup>-/-</sup>SOCS1<sup>-/-</sup> CD4<sup>+</sup> T cells by real-time RT-PCR (Fig. 5C). The SOCS3 levels were 1.5–2 times higher in naïve SOCS1<sup>del/del</sup> T cells than control T cells. Higher SOCS3 levels in naïve SOCS1<sup>del/del</sup> T cells were also shown by Western blotting (Fig. 5A). In addition, SOCS3 levels were significantly lower in IFN-γ<sup>-/-</sup> and IFN-γ<sup>-/-</sup>SOCS1<sup>-/-</sup> T cells (Fig. 5C). SOCS1 levels were also low in IFN-γ<sup>-/-</sup> T cells (Fig. 5C). These results indicate that SOCS3 as well as SOCS1 levels in naïve T cells are positively regulated by IFN-γ.

To elucidate the molecular mechanism of SOCS3 elevation in SOCS1-deficient cells, IFN-γ-mediated SOCS3 induction was compared between WT and STAT1-deficient (STAT1<sup>-/-</sup>) mouse embryonic fibroblasts (MEFs) (Fig. 5D). SOCS3 up-regulation was not observed in STAT1<sup>-/-</sup> MEFs, suggesting that IFN-γ-mediated STAT1 activation led to the elevation of SOCS3 levels as we have shown before (40).

**Suppression of Th17 differentiation by forced expression of SOCS3 in T cells**

To confirm our hypothesis that SOCS3 negatively regulates STAT3-mediated Th17 differentiation, we used T cell-specific SOCS3 Tg mice. We have shown that Th2 is preferentially induced in this SOCS3 Tg mouse (30). TCR signaling has also been shown to be down-modulated in SOCS3 Tg T cells (41). However, Th17 differentiation has not been examined in SOCS3 Tg T cells. Real-time RT-PCR analysis showed that endogenous SOCS3 levels in naïve CD4<sup>+</sup> T cells from SOCS3 Tg mice were approximately four times higher than those in WT littermate T cells (Fig. 6A). As expected, IL-6-induced STAT3 phosphorylation was reduced in SOCS3 Tg T cells compared with WT T cells (Fig. 6B). FACS and ELISA revealed that SOCS3 Tg CD4<sup>+</sup> T cells differentiate into Th1 cells less efficiently than those from WT littermates (Fig. 6C). Unlike SOCS1<sup>del/del</sup> T cells, we did not see a strong differentiation into Th1 in SOCS3 Tg CD4<sup>+</sup> T cells under the Th17 differentiation condition (Fig. 6C). As shown in Fig. 6D, EAE onset was significantly delayed in SOCS3 Tg mice. CD4<sup>+</sup> T cells from SOCS3 Tg mice were shown to preferentially differentiate into Th2 under OVA/alum immunization conditions (30). However, we could not detect high levels of IL-4 in SOCS3 Tg mice under MOG peptide/CFA immunization conditions, probably because CFA adjuvant could not induce Th2 efficiently (data not shown). Taken together, these data support our hypothesis that the induction of SOCS3 in T cells suppressed Th17 differentiation and EAE onset by restricting STAT3 activation.

**Impaired TGF-β-mediated signals in SOCS1-deficient cells**

Up-regulation of SOCS3 could not explain hyper-Th1 polarization of SOCS1<sup>del/del</sup> T cells. TGF-β plays an essential role in Th17 differentiation and TGF-β has been shown to suppress Th1 differentiation (42). Therefore, we hypothesized that TGF-β signaling was impaired in SOCS1-deficient T cells. We first compared the
FIGURE 7. Reduced TGF-β effects in SOCS1-deficient cells. A, TGF-β-mediated Th1 suppression in SOCS1<sup>−/−</sup> T cells (left panel) and SOCS3 Tg T cells (right panel). Naive CD4<sup>+</sup>CD25<sup>−</sup> T cells were activated in vitro by 1 μg/ml anti-TCR mAb, 0.5 μg/ml anti-CD28 mAb, and mIL-12p70 10 ng/ml with or without the indicated concentrations of hTGF-β1 for 5 days. Then, cells were collected and restimulated by 1 μg/ml anti-TCR mAb for 16 h. IFN-γ concentrations in the supernatants were determined by ELISA. One representative data from two independent experiments are shown.

B, Suppression of RORγt induction in SOCS1<sup>−/−</sup> T cells under the Th17 condition. Total RNA was extracted from CD4<sup>+</sup> T cells activated in vitro for 16 h with 1 μg/ml anti-TCR mAb, 0.5 μg/ml anti-CD28 mAb, and 10 ng/ml hIL-6 in the presence of the indicated concentrations of hTGF-β1 in the absence (left panel) or presence (middle panel) of 100 U/ml mIFN-γ. Similar experiments were performed using T cells from WT (IFN-γ<sup>−/−</sup>/SOCS1<sup>−/−</sup>), IFN-γ<sup>−/−</sup>/SOCS3<sup>−/−</sup> and IFN-γ<sup>−/−</sup>/SOCS1<sup>−/−</sup>/STAT1<sup>−/−</sup> mice (right panel). RORγt mRNA levels were determined by real-time RT-PCR. One representative data from two independent experiments are shown.

C, Smad7 expression under the Th17 condition. Primary CD4<sup>+</sup> T cells from the indicated mice were stimulated with 1 μg/ml anti-TCR mAb, 10 ng/ml hIL-6, and 1 ng/ml hTGF-β1 for the indicated periods. RT-PCR (upper panel) and real-time RT-PCR (lower panel) of the indicated genes were performed. One representative data from two independent experiments are shown.

D, SBE reporter activity in primary T cells. WT and SOCS1<sup>−/−</sup> primary CD4<sup>+</sup> T cell were transfected with SBE-luciferase reporter plasmids by electroporation (see Materials and Methods) and stimulated by 2 ng/ml hTGF-β1 in the presence or absence of pretreatment of 100 U/ml mIFN-γ for 1 h. After 8 h, cells were collected and luciferase

E, Inhibitory activity (%).
effect of TGF-β on Th1 differentiation between Ct (WT) and SOCS1<sup>del/del</sup> T cells. As shown in Fig. 7A, left, TGF-β strongly suppressed IFN-γ production in Cd4<sup>+</sup> T cells, while SOCS1<sup>del/del</sup> T cells were resistant to suppressive effect of TGF-β. This was not observed in SOCS3 Tg T cells, indicating that TGF-β resistance in SOCS1-deficient T cells was SOCS3 independent (Fig. 7A, right).

These data suggest that strong Th1 polarization of SOCS1<sup>del/del</sup> T cells in the presence of IL-6 and TGF-β is partly due to impaired TGF-β signaling.

Then, we examined the effect of SOCS1 deficiency on TGF-β-mediated RORγt up-regulation. RORγt mRNA induction in naïve Cd4<sup>+</sup> T cells was determined by real-time RT-PCR at 16 h after stimulation with anti-TCR Ab and IL-6 in the presence of various concentrations of TGF-β. As shown in Fig. 7B, left, TGF-β strongly enhanced RORγt expression in a dose-dependent manner in WT (control) T cells. However, TGF-β-mediated RORγt up-regulation was severely impaired in SOCS1<sup>del/del</sup> T cells. Exogenous IFN-γ partially suppressed RORγt expression in WT T cells in the presence of a high dose of IFN-γ; however, RORγt induction was still suppressed in SOCS1<sup>del/del</sup> T cells (Fig. 7B, middle). However, RORγt induction was similar between IFN-γ<sup>−/−</sup> SOCS1<sup>−/−</sup> and IFN-γ<sup>−/−</sup>/SOCS1<sup>−/−</sup> T cells (Fig. 7B, right).

These data indicated that a strong difference in RORγt induction between SOCS1-deficient T cells and WT T cells was dependent on endogenous IFN-γ secreted from the SOCS1-deficient T cell itself. Thus, strong suppression of RORγt induction in SOCS1-deficient T cells was due to a strong suppression of TGF-β signaling by enhanced IFN-γ signaling. In other words, SOCS1 is necessary for proper TGF-β signaling by protecting cells from the strong antagonistic effect of IFN-γ.

Smad7 has been proposed to be the mechanism of the antagonistic effect of IFN-γ against TGF-β (43, 44). However, as shown in Fig. 7C, Smad7 expression was not different between WT and SOCS1<sup>del/del</sup> T cells regardless of IFN-γ.

Then, we investigated Smad transcription factor activity, since Smads play essential roles in biological functions of TGF-β. Smad transcriptional activity was assessed by the SBE reporter assay (34). Primary Cd4<sup>+</sup> T cells were transfected with the SBE reporter plasmid by electroporation, then luciferase activity was measured after T cells were stimulated with TGF-β<sup>−/−</sup>/IFN-γ. As shown in Fig. 7D, TGF-β strongly enhanced SBE reporter activity, but was repressed by IFN-γ in WT T cells, whereas SBE reporter activity remained very low in SOCS1<sup>del/del</sup> T cells in response to TGF-β (Fig. 7D). Taken together with the impaired induction of RORγt, these data indicate that Smad transcriptional activity was strongly suppressed in SOCS1-deficient T cells.

To confirm that SOCS1 is necessary to prevent too strong of an antagonistic effect of IFN-γ against Smad transcription activity, we used SOCS1-deficient (SOCS1<sup>−/−</sup>) and STAT1-deficient (STAT1<sup>−/−</sup>) MEFs, since MEFs do not produce endogenous IFN-γ (Fig. 7E). IFN-γ suppressed TGF-β-mediated Smad transcriptional activity to <50% levels in WT MEFs, while it was suppressed to <20% levels in SOCS1-deficient MEFs. Thus, IFN-γ has a much stronger antagonistic effect on TGF-β signaling in SOCS1-deficient MEFs as well as SOCS1-deficient T cells. In contrast, IFN-γ-mediated Smad suppression was rarely observed in STAT1<sup>−/−</sup> MEFs. In addition, overexpression of SOCS1 inhibited the IFN-γ effect, whereas overexpression of SOCS3 did not affect the antagonistic IFN-γ effect. These data suggest that suppression of Smad activity by IFN-γ is dependent on STAT1 but not on SOCS3. Furthermore, STAT1 phosphorylation is stronger in SOCS1<sup>−/−</sup> MEFS than in WT MEFS, although TGF-β-mediated Smad2 phosphorylation was not different between WT and SOCS1<sup>−/−</sup> MEFS (data not shown). These data also suggested that TGF-β-mediated Smad transcriptional activity was strongly suppressed in SOCS1<sup>−/−</sup> cells because of a hyperactivation of STAT1.

**Discussion**

In this study, we have shown that SOCS1 is an important regulatory factor that directs the differentiation of Th1 and Th17. Following ligation of the Ag receptor, STAT3 activated by IL-6 as well as IL-23 and IL-21 and TGF-β together induce the transcription of RORγt, which, in turn, participates in and may be sufficient for the induction of IL-17 expression (12, 13, 17). Th17 differentiation is blocked at an early stage by IFN-γ and IL-4, probably because IL-6 as well as TGF-β signals are antagonized by IFN-γ and/or IL-4 (6, 7). However, the molecular mechanism for these suppressions has not been clarified. We propose that SOCS1 is an important determinant for these processes. In Fig. 8, we propose a model of STAT1-mediated negative regulation of Th17 differentiation (RORγt induction) by STAT3 and Smads. We have shown that SOCS3 is probably a part of the mechanism of STAT1-mediated suppression of STAT3; however, an unknown mechanism (X) other than SOCS3 is involved in the suppression of Smad transcriptional activity. SOCS1 is necessary for Th17 development because SOCS1 needs to prevent too strong antagonistic signals of IFN-γ/STAT1 against STAT3 and Smads.

IFN-γ has been shown to suppress the differentiation of Th17 cells by reducing IL-23R expression on Cd4<sup>+</sup> T cells (8). It is not known, however, how IFN-γ inhibits IL-23R expression. Because IL-23R expression is induced by the IL-6/STAT3/RORγt pathway (17), STAT1 activated by IFN-γ may antagonize STAT3 activated by IL-6. Likewise, IFN-γ/STAT1 and IL-4/STAT6 antagonize each other in the differentiation of Th1 and Th2 cells. The molecular basis of such cross-suppression, the suppression of one STAT by other STATs has not been established. Multiple mechanisms

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**FIGURE 8.** Schematic model for IFN-γ-mediated suppression of Th17 induction. Activated STAT1 by IFN-γ, which is usually inhibited by SOCS1, suppresses RORγt induction by suppressing IL-6-mediated STAT3 and TGF-β-mediated Smad activities. SOCS3 up-regulated by STAT1 is a mechanism for the suppression of STAT3 activation. However, SOCS3 is not involved in the suppression of TGF-β-mediated Smad activation. Therefore, an unknown factor (X) may suppress Smad activity.
have been suggested, including heterodimer formation between different STATs, repression of transcriptional activities, and induction of SOCS proteins (22). SOCS1 is induced by both IFN-γ and IL-4; therefore, early cytokines may suppress late cytokines by inducing SOCS1 (45). SOCS1 induced by IL-6 has been shown to be involved in the IL-6-mediated suppression of IFN-γ-induced STAT1 activation in Th1 cells (46).

We noticed that IL-6-induced STAT3 activation was severely repressed in SOCS1del/del T cells, but not in the IFN-γ−/− background (Fig. 5, A and B). Similarly, LIF-induced STAT3 activation was repressed in IFN-γ−/−pretreated SOCS1−/− MEFs, but not in the SOCS3−/− background (data not shown). Therefore, we suspected that SOCS3 induced by IFN-γ is involved in the STAT3 repression in T cells like in MEFs. We confirmed that SOCS3 levels were more increased in SOCS1del/del T cells and were extremely reduced in the IFN-γ−/− background (Fig. 5, A and C). In addition, we showed that SOCS3 levels are higher in SOCS1−/− cells than WT cells. Therefore, increased levels of SOCS3 in SOCS1del/del T cells and low levels of SOCS3 in IFN-γ−/− T cells can explain reduced and enhanced STAT3 activation, respectively. Consistent with these data, prior expression of SOCS3 in T cells of Tg mice suppressed IL-6-induced STAT3 activation, Th17 differentiation, and EAE development (Fig. 6). In contrast, SOCS3-deficient T cells showed a clear enhancement of Th17 development and STAT3 activation induced by either IL-23 or IL-6 plus TGF-β (16, 28) and T cell-specific SOCS3 cKO mice showed earlier onset and exacerbation of EAE (K. Tanaka, unpublished data).

However, we do not think that SOCS3 is the sole mechanism of reduction of Th17 differentiation in SOCS1del/del T cells as well as the antagonistic effect of IFN-γ on Th17 differentiation. For example, SOCS3 Tg mice showed a delayed onset of EAE, but severe EAE was developed at a late stage, whereas EAE was strongly suppressed in SOCS1 cKO mice even at a late stage (cf. Figs. 2A and 6D). Unlike T cell-specific SOCS1 cKO mice, we did not observe hyper-Th1 polarization in SOCS3 Tg mice. Therefore, suppression of TGF-β signaling in SOCS1del/del T cells might be another mechanism for suppression of Th17 and strong induction of Th1.

The precise role of TGF-β on Th17 differentiation has not been clarified. It has been shown to suppress Th1 and Th2 differentiation. However, Abs against IFN-γ, IL-12, and IL-4 could not replace TGF-β (9). It has not been clarified whether Smads are critical for TGF-β-mediated Th17 differentiation. Therefore, we thought that regulation of Smad transcriptional activity by Th17-antagonistic cytokines is another important mechanism for Th17 regulation. We propose that IFN-γ/STAT1 antagonizes Smads activated by TGF-β, which is strongly enhanced by SOCS1 deficiency.

As shown in Fig. 7, TGF-β-mediated Th1 suppression was not observed in SOCS1del/del T cells. This is partly because TGF-β signaling is suppressed not only by high levels of IFN-γ but also by hyper-STAT1 activation in SOCS1del/del T cells. It has been shown that induction of Smad7, which inhibits Smad phosphorylation by interacting with the TGF-β receptor, is a mechanism of the suppression of TGF-β signaling by IFN-γ (43, 44). However, we do not think this is the case, since we could not observe strong Smad7 induction nor suppression of Smad2 phosphorylation in SOCS1del/del cells. Another important mechanism has been suggested that STAT1 activation represses Smad transcriptional activity by limiting amounts of coactivator, p300/CPB (47). Using MEF cells, we demonstrated that the IFN-γ/STAT1-mediated suppression of Smad activity was extremely strong in SOCS1−/− cells (Fig. 7E) without affecting Smad2 phosphorylation (data not shown), whereas, in STAT1−/− cells, IFN-γ-mediated suppression was abrogated (Fig. 7E). Therefore, our data suggest that STAT1 suppresses TGF-β signaling while SOCS1 enhances TGF-β signaling by repressing STAT1. Because SOCS1−/− T cells produce high amounts of IFN-γ (Fig. 7A), Smad transcriptional activity must be severely repressed in SOCS1del/del T cells without supplying exogenous IFN-γ (Fig. 7D). The precise molecular mechanism for STAT1-mediated Smad suppression is under investigation.

Importantly, we still do not know anything about the role of Smads on Th17 differentiation. SOCS1-deficient T cells could facilitate the discovery of the target genes of Smads for Th17 development and the novel mechanisms for the suppression of Smad activities by STAT1.

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Disclosures

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