SOCS1 is essential for regulatory T cell functions by preventing loss of Foxp3 expression as well as IFN-γ and IL-17A production

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Regulatory T cells (T_{reg} cells) maintain immune homeostasis by limiting inflammatory responses. SOCS1 (suppressor of cytokine signaling 1), a negative regulator of cytokine signaling, is necessary for the suppressor functions of T_{reg} cells in vivo, yet detailed mechanisms remain to be clarified. We found that *Socs1*$^{-/-}$ T_{reg} cells produced high levels of IFN-γ and rapidly lost Foxp3 when transferred into *Rag2*$^{-/-}$ mice or cultured in vitro, even though the CNS2 (conserved noncoding DNA sequence 2) in the Foxp3 enhancer region was fully demethylated. *Socs1*$^{-/-}$ T_{reg} cells showed hyperactivation of STAT1 and STAT3. Because Foxp3 expression was stable and STAT1 activation was at normal levels in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells, the restriction of IFN-γ–STAT1 signaling by SOCS1 is suggested to be necessary for stable Foxp3 expression. However, *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells had hyperactivated STAT3 and higher IL-17A (IL-17) production compared with *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ T_{reg} cells and could not suppress colitis induced by naive T cells in *Rag2*$^{-/-}$ mice. In vitro experiments suggested that cytokines produced by *Socs1*$^{-/-}$ T_{reg} cells and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells modulated antigen-presenting cells for preferential Th1 and Th17 induction, respectively. We propose that SOCS1 plays important roles in T_{reg} cell integrity and function by maintaining Foxp3 expression and by suppressing IFN-γ and IL-17 production driven by STAT1 and STAT3, respectively.

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Abbreviations used: cKO, conditional KO; iT_{reg} cell, inducible T_{reg} cell; nT_{reg} cell, natural T_{reg} cell.

A variety of pathologies of autoimmune diseases and allergic diseases are caused by the immune responses to self, environmental nonmicrobial antigens, and infectious agents. Regulatory T cells (T_{reg} cells), which are characterized by expression of the Forkhead transcription factor, Foxp3, play an indispensable role in immunological tolerance, protecting the host from excessive immune responses (Hori et al., 2003; Sakaguchi, 2004; Sakaguchi et al., 2008; Belkaid and Tarbell, 2009). Foxp3 plays an essential role in the suppressive function of T_{reg} cells (Wan and Flavell, 2007), and Foxp3 deficiency causes a multiorgan autoimmune disease as can be observed in the scurfy mouse and in patients with IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome; Bennett and Ochs, 2001; Bennett et al., 2001; Brunkow et al., 2001). Foxp3 induction in natural T_{reg} cells (nT_{reg} cells) occurs in vivo during thymic differentiation, under the influence of relatively high avidity interactions of the TCR with self-antigens.

Although the suppression of autoimmunity by T_{reg} cells is now well established, recently, nT_{reg} cells have been shown to convert to effector/helper T cells (Komatsu et al., 2009). Although most T_{reg} cells retain high Foxp3 expression after adoptive transfer to a nonpathogenic setting, 10–15% of T_{reg} cells were found to lose Foxp3 expression after adoptive transfer

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 208 No. 10 2055-2067
www.jem.org/cgi/doi/10.1084/jem.20110428

The Journal of Experimental Medicine

into lymphopenic hosts. A recent study showed that half of T_{reg} cells transferred into lymphopenic hosts did not die, but rather began producing IL-2 and IFN-γ (Komatsu et al., 2009). Additionally, multiple recent studies have demonstrated that in the inflammatory settings of autoimmunity, there is a loss of Foxp3 during inflammatory responses (Zhou et al., 2009; Murai et al., 2010). The adoptive transfer of T_{reg} cells into *Cd3ε*$^{-/-}$ hosts, which retain B lymphocytes, resulted in the loss of Foxp3 expression and the generation of lapsed T_{reg} cells that differentiated into follicular helper T cells in Peyer's patches that promoted IgA class switching (Tsuji et al., 2009). Such exFoxp3 cells (Zhou et al., 2009) or lapsed T_{reg} cells (Murai et al., 2010) develop an effector-memory phenotype, produce pathogenic cytokines, and can trigger the development of autoimmunity. Two possibilities were proposed regarding the developmental plasticity of T_{reg} cells: (1) the lineage reprogramming of the conversion of committed Foxp3^{+} cells to Foxp3^{-} cells or (2) the expansion of uncommitted T_{reg} cells, which easily lose Foxp3 (Hori, 2010). In any case, the molecular basis for such T_{reg} cell conversion and the signals that ensure the stability of T_{reg} cells have not yet been clarified. On the contrary, recent work by Rubtsov et al. (2010) reported that highly purified T_{reg} cells were very stable under physiological and inflammatory conditions. Such clarification is also necessary for the development of applications for transferring T_{reg} cells to treat autoimmune diseases or to prevent rejections of transplantations.

SOCS1 (suppressor of cytokine signaling 1) is apparently defined as an important mechanism for the negative regulation of the cytokine–JAK–STAT pathway (Yoshimura et al., 2007), and uncontrolled IFN-γ signaling results from a deficiency of SOCS1. SOCS1 is highly expressed in T_{reg} cells (Lu et al., 2009). It has been reported that SOCS1 expression is reduced in lupus-affected (NZB × NZW) F1 mice (Sharabi et al., 2009), and expression levels of SOCS1 are altered in patients with rheumatoid arthritis or systemic lupus erythematosus (Isomäki et al., 2007; Chan et al., 2010). Analyses of T cell–specific *Socs1* conditional KO (cKO; *Lck*Cre-*Socs1*$^{f/f}$) mice revealed that SOCS1-deficient effector T cells produce high levels of IFN-γ and low levels of IL-17A (IL-17; Tanaka et al., 2008). Although we have shown that the number of Foxp3-positive T_{reg} cells in cKO mice is higher than that in WT mice (Lu et al., 2009), SOCS1-deficient T_{reg} cells lost proper suppression functions in T_{reg} cell–specific *Socs1*-cKO mice (Lu et al., 2010). However, detailed mechanisms for the impaired T_{reg} cell function by SOCS1 deficiency remain to be clarified.

This study was undertaken to clarify the role of SOCS1 in the stability and suppressive function of T_{reg} cells. We observed autoimmune phenotypes in T cell–specific *Socs1*-cKO mice, suggesting a defective T_{reg} cell function in these mice. The defective suppression activity of SOCS1-deficient T_{reg} cells was confirmed through the failure to suppress colitis in *Rag2*$^{-/-}$ mice by the cotransfer of naive T cells and T_{reg} cells. In lymphopenic conditions, *Socs1*$^{-/-}$ T_{reg} cells tended to lose Foxp3 and were easily converted into Th1-like IFN-γ–producing cells. Foxp3 levels were preserved in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells, yet *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells could still not suppress colitis induced by the transfer of naive T cells. To our surprise, *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells produced extremely high levels of IL-17, thereby instructing Th17 cell differentiation of naive T cells in *Rag2*$^{-/-}$ mice. *Il-17*$^{-/-}$ naive T cells still caused colitis but were suppressed by the cotransfer of *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells, which suggests that *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells instruct naive T cells to become colitogenic by producing high levels of IL-17. In vitro experiments suggest that cytokines from *Socs1*$^{-/-}$ T_{reg} cells play roles in the instruction of Th1 and Th17 through APCs. We propose that SOCS1 is a guardian of T_{reg} cells because SOCS1 inhibits the conversion of T_{reg} cells into Th1- and Th17-like IFN-γ– and IL-17–producing cells.

RESULTS

Autoimmune phenotypes caused by *Lck*Cre-*Socs1*$^{f/f}$ mice and impaired suppression activity of SOCS1-deficient nT_{reg} cells in vivo

As reported previously, T cell–specific *Socs1*-deleted (*Lck*-Cre-*Socs1*$^{f/f}$; cKO) mice survived >6 mo (Tanaka et al., 2008), yet all of these mice eventually developed dermatitis, splenomegaly, and lymphadenopathy with age (Fig. 1, A–C). Inflammatory cellular infiltrations in the skin, liver, and kidney were observed in these mice (Fig. 1 D). Serum Ig levels were much higher in cKO mice than in *Socs1*$^{f/f}$ (WT) mice, and dsDNA antibodies were detected as well (Fig. 1 E). These data suggest that cKO mice developed a systemic inflammatory and autoimmune disease.

The autoimmune phenotype of T cell–specific *Socs1*-cKO mice has been thought to be mostly caused by hyperactivation of effector T cells and the production of a huge amount of IFN-γ (Fig. 1 F). Because *Ifnγ*$^{-/-}$*Lck*Cre-*Socs1*$^{f/f}$ mice did not develop dermatitis (not depicted), these phenotypes were dependent on IFN-γ. cKO mice and *Socs1*$^{f/f}$ littermates were crossed with knockin mice with Foxp3-IRES-GFP as reported previously (Wang et al., 2008), and the number of nT_{reg} cells was increased in cKO-*Foxp3*^{GFP} mice in the thymus (Fig. 1 G) and periphery (Zhan et al., 2009). Similar autoimmune phenotypes were observed in T_{reg} cell–specific *Socs1*-deleted mice, *Foxp3*Cre-*Socs1*$^{f/f}$ mice (Lu et al., 2010), suggesting that SOCS1-deficient nT_{reg} cells have a defective suppression activity.

The defective suppression activity of SOCS1-deficient T_{reg} cells in vivo was confirmed by the failure to suppress colitis in *Rag2*$^{-/-}$ mice by the cotransfer of naive T cells (Ly5.1) and T_{reg} cells (Ly5.2; Fig. 2 A and Fig. S1). T_{reg} cells were purified by FACS sorting as CD3^{+}CD4^{+}CD25^{+}GFP^{+} (>99% Foxp3 positive). 4 wk after transfer, the fraction of Foxp3-positive cells from Ly5.2-positive cKO-*Foxp3*^{GFP} mice was significantly decreased compared with those from Ly5.2-positive WT-*Foxp3*^{GFP} mice (Fig. 2 B). IFN-γ levels in the mesenteric LN in *Rag2*$^{-/-}$ mice cotransferred with naive T cells and *Socs1*$^{-/-}$ T_{reg} cells were much higher than in *Rag2*$^{-/-}$ mice transferred with naive T cells and WT T_{reg} cells (not depicted). These results indicate that the suppressive function

of SOCS1-deficient T_{reg} cells was impaired in lymphopenic conditions, concomitant with faster loss of Foxp3 expression, compared with that of WT T_{reg} cells.

Loss of Foxp3 expression and conversion to Th1-like cells of SOCS1-deficient T_{reg} cells in lymphopenic conditions

We then investigated the fate of $Foxp3^+$ cells in lymphopenic conditions. T_{reg} cells from WT-*Foxp3*^{GFP} or cKO-*Foxp3*^{GFP} mice were transferred into *Rag2*$^{-/-}$ mice, and 6 wk later, Foxp3 positivity was examined by flow cytometry. WT T_{reg} cells lost Foxp3 expression in the single-transfer condition more significantly than in the cotransfer with naive T cells, yet they did not develop colitis, as shown previously (Komatsu et al., 2009). Surprisingly, SOCS1-deficient T_{reg} cells transferred into *Rag2*$^{-/-}$ mice developed colitis (Fig. 2 C and Fig. S2). SOCS1-deficient T_{reg} cells experienced a more profound loss than did WT T_{reg} cells (63.1% $Foxp3^+$ in WT T_{reg} cells vs. 40.7% in SOCS1-deficient T_{reg} cells; Fig. 2 D).

Not only Foxp3 but also other phenotypical markers of T_{reg} cells, CD25 and CTLA4, which have been shown to be critical molecules in the suppression activity of T_{reg} cells (Wing et al., 2008), were altered in transferred SOCS1-deficient T_{reg} cells. Before transfer, SOCS1-deficient T_{reg} cells expressed CD25 and CTLA4 at significantly higher levels than did WT T_{reg} cells. After transfer, however, CD25 and CTLA4 were more profoundly down-regulated in *Socs1*$^{-/-}$ T_{reg} cells than in WT T_{reg} cells, in both the Foxp3-maintaining fraction ($Foxp3^{+\rightarrow+}$; Fig. 2 E) and the exFoxp3 fraction ($Foxp3^{+\rightarrow-}$; Fig. 2 E, top and bottom). In addition, transferred *Socs1*$^{-/-}$ T_{reg} cells produced higher amounts of IFN-γ from both the Foxp3-maintaining and the Foxp3-losing fractions (Fig. 2 F, top and bottom). The Foxp3-maintaining fraction of SOCS1-deficient $Foxp3^+$ T_{reg} cells also produced IL-2 in addition to IFN-γ (Fig. 2 G, top and bottom). These results indicate that SOCS1-deficient T_{reg} cells tend to lose Foxp3 expression and produce effector cytokines, thus suggesting that SOCS1 is necessary for preventing IFN-γ–producing exFoxp3 cell development.

To exclude the possibility that the higher number of $Foxp3^-$ cells in $Foxp3^+$*Socs1*$^{-/-}$ T_{reg} cell transfer was caused by an extraordinary expansion of contaminated $Foxp3^-$*Socs1*$^{-/-}$ cells, activated/memory *Socs1*$^{-/-}$ or *Socs1*$^{+/+}$ T cells ($Ly5.2^+$ $CD44^{high}CD62L^-Foxp3^-$ cells) were mixed to 3.3% in the cotransfer of Ly5.1 WT naive T cells and T_{reg} cells (Fig. S3, top) or to 5% in the transfer of Ly5.1 WT T_{reg} cells into *Rag2*$^{-/-}$ mice (Fig. S3, bottom). Any abnormal expansion of activated/memory *Socs1*$^{-/-}$ T cells was not observed, suggesting that the larger number of $Foxp3^-$ cells in *Socs1*$^{-/-}$ T_{reg} cell transfer was not caused by rapid expansion of contaminated SOCS1-deficient $Foxp3^-$ T cells.

IL-10 from T_{reg} cells has been shown to play an important role in the suppression of naive T cell–induced colitis in *Rag2*$^{-/-}$ mice (Maloy et al., 2003). Thus, we suspected that impaired IL-10 production from SOCS1-deficient T_{reg} cells could be a mechanism for SOCS1-deficient T_{reg} cell–mediated colitis. However, production of IL-10 was significantly higher in *Socs1*$^{-/-}$ T_{reg} cells compared with WT T_{reg} cells both before and after transfer, suggesting that IL-10 is not involved in the SOCS1-deficient T_{reg} cell–mediated colitis (Fig. S4).

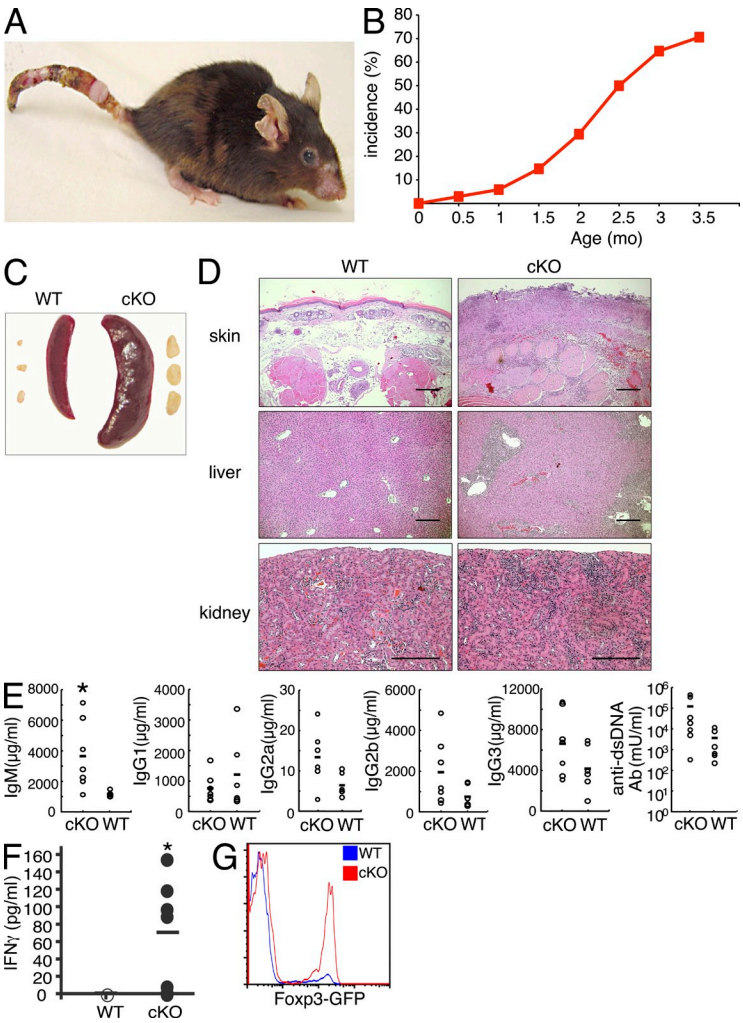

Figure 1. Characterization of *Lck*Cre-*Socs1*$^{f/f}$ (cKO) mice. (A) Representative appearance of a 12-wk-old cKO mouse. (B) Incidence of dermatitis in cKO mice ($n = 20$). (C) Representative macroscopic view of the spleen and LN of a *Socs1*$^{f/f}$ (WT) mouse and its age (12 wk old)- and sex-matched cKO counterpart. (D) Representative histopathology of the skin, liver, and kidneys of a WT mouse and its age (12 wk old)- and sex-matched cKO counterpart. Data are representative of five independent experiments. Bars, 200 μm. (E and F) Serum titers of Ig subclasses and anti-dsDNA antibodies (E) and serum levels of IFN-γ (F) from WT and cKO mice measured by ELISA. Each symbol indicates individual host mouse (*, $P < 0.05$). Data are representative of seven independent experiments. Horizontal bars indicate the mean. (G) Representative Foxp3-GFP expression on $CD3^+CD4^+$ T cells from the thymus of WT and cKO mice as determined by flow cytometry.

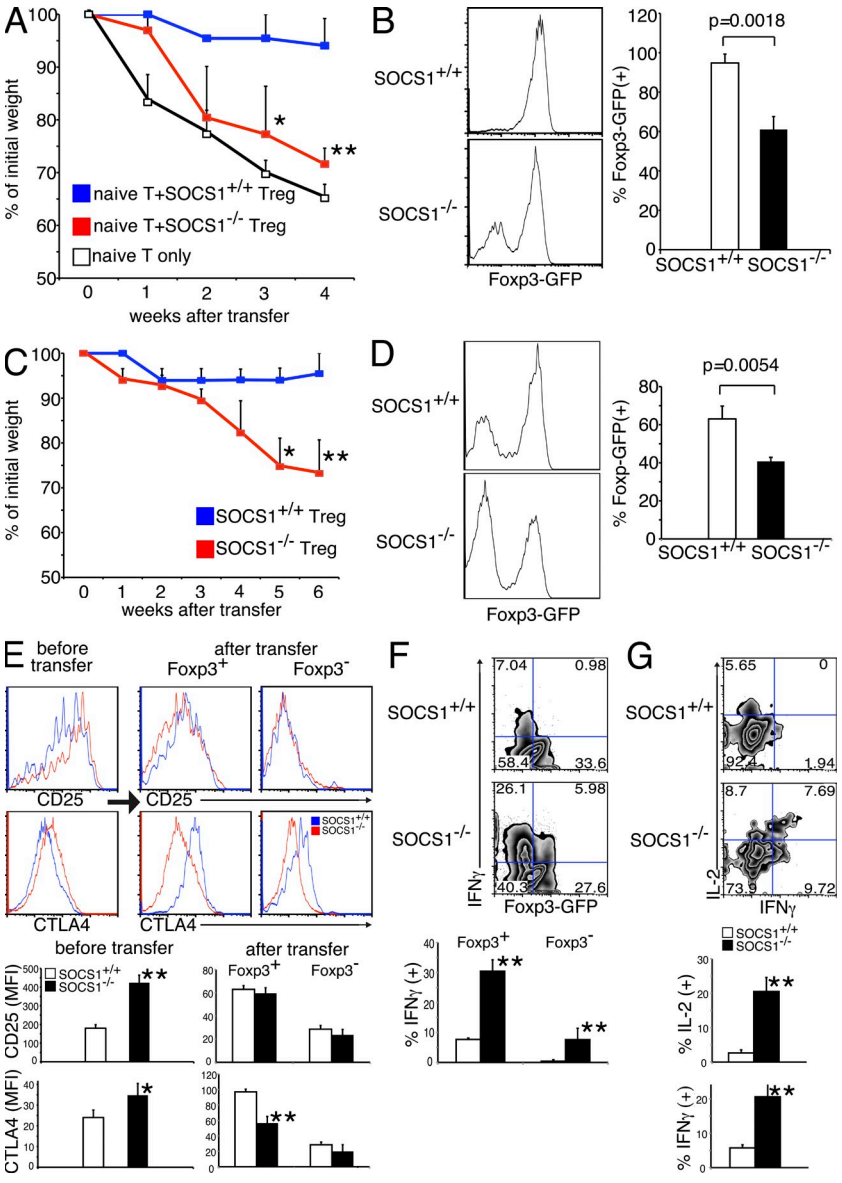

Figure 2. SOCS1-deficient T_{reg} cells have attenuated suppressive function, lose Foxp3 expression, and produce effector cytokines. (A and B) 2×10^5 T_{reg} cells from the LN of Ly5.2 WT-*Foxp3*^{GFP} mice (*Socs1*$^{+/+}$ T_{reg} cells) or Ly5.2 cKO-*Foxp3*^{GFP} mice (*Socs1*$^{-/-}$ T_{reg} cells) were cotransferred with 4×10^5 Ly5.1 naive T cells into *Rag2*$^{-/-}$ mice. 4 wk later, recipient mice were analyzed. (A) Body weight changes. (B) Flow cytometric analysis of Foxp3-GFP expression on Ly5.2$^+$CD3$^+$CD4$^+$ T cells from the LN in *Rag2*$^{-/-}$ mice (percentage of Foxp3-GFP$^+$ shown in bar graph). (C–G) 2×10^5 *Socs1*$^{+/+}$ or *Socs1*$^{-/-}$ Foxp3$^+$ T_{reg} cells were transferred into *Rag2*$^{-/-}$ mice. 6 wk after transfer, recipient mice were analyzed. (C) Body weight changes. (D) Flow cytometric analysis of Foxp3-GFP expression on CD3$^+$CD4$^+$ T cells from the LN in *Rag2*$^{-/-}$ mice (percentage of Foxp3-GFP$^+$ indicated in bar graph). (E) Expression profiles of CD25 and intracellular CTLA4 on CD3$^+$CD4$^+$GFP$^+$ cells and GFP$^-$ cells from the LN in *Rag2*$^{-/-}$ mice (right) compared with those on CD3$^+$CD4$^+$GFP$^+$ cells before transfer (left; mean fluorescent intensity [MFI], bottom). (F) Flow cytometric analysis of Foxp3-GFP and IFN-γ expression on CD3$^+$CD4$^+$cells from the LN in *Rag2*$^{-/-}$ mice (percentage of IFN-γ$^+$ shown in bar graph below). (G) Expression of IFN-γ and IL-2 on CD3$^+$CD4$^+$GFP$^+$ cells from the LN in *Rag2*$^{-/-}$ mice (percentage of IL-2– and IFN-γ–producing cells indicated in bar graph). (A–G) Data are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.01$). Values represent the mean ± SD.

Rapid methylation of the CNS2 (conserved noncoding DNA sequence 2) region in the Foxp3 enhancer of committed SOCS1-deficient T_{reg} cells

Like in *Rag2*$^{-/-}$ mice, Foxp3 levels were decreased more rapidly in *Socs1*$^{-/-}$ T_{reg} cells than in WT T_{reg} cells in vitro. CFSE dilution revealed that the loss of Foxp3 occurred mostly in divided T_{reg} cells, suggesting that the loss of Foxp3 is associated with cell division or that cells that lost Foxp3 expression divide faster than Foxp3$^+$ cells (Fig. 3 A). It has been reported that CD4$^+$CD25$^+$CD62L^{high} T_{reg} cells maintained stable Foxp3 expression along with suppressive activity after in vitro expansion compared with CD4$^+$CD25$^+$CD62L^{low} T_{reg} cells (Scalapino and Daikh, 2009). Thus, we compared Foxp3 expression levels in *Socs1*$^{+/+}$ and *Socs1*$^{-/-}$ T_{reg} cells between the CD62^{high} and CD62L^{low} fraction after 3-d in vitro culture. The fraction of CD62L^{low} was much higher than that of CD62L^{high} in *Socs1*$^{-/-}$ T_{reg} cells (Fig. 3 B). As reported, Foxp3 expression was decreased in the CD62L^{low} fraction in WT T_{reg} cells, and Foxp3 was maintained in the CD62L^{high} fraction (Fig. 3 C, left). In *Socs1*$^{-/-}$ T_{reg} cells, Foxp3 was also maintained in the CD62L^{high} fraction. However, the loss of Foxp3 expression in the CD62L^{low} fraction was significantly more drastic in *Socs1*$^{-/-}$ T_{reg} cells than in WT T_{reg} cells (Fig. 3 C, right).

Recently, it has been reported that the DNA methylation status of the CNS2 region in the Foxp3 enhancer is particularly important for maintenance of Foxp3 expression (Floess et al., 2007; Kim and Leonard, 2007; Zheng et al., 2010). To examine whether SOCS1-deficient Foxp3$^+$ T_{reg} cells contained uncommitted T_{reg} cells or unstable inducible T_{reg} cells (iT_{reg} cells), we determined the methylation status of the CNS2 region in SOCS1-deficient T_{reg} cells. As shown in Fig. 3 D, the CNS2 region of T_{reg} cells from the LN of SOCS1-cKO mice were fully demethylated, similar to those of WT mice (Fig. 3 D). This indicates that >95% SOCS1-deficient T_{reg} cells were committed and did not contain iT_{reg} cells. After 3-d in vitro culture, the Foxp3-maintaining fraction (Foxp3$^{+\rightarrow+}$) retained full demethylation in the CNS2 region in both *Socs1*$^{+/+}$ and *Socs1*$^{-/-}$ T_{reg} cells. However, the CNS2 region in the exFoxp3 fraction (Foxp3$^{+\rightarrow-}$) was fully

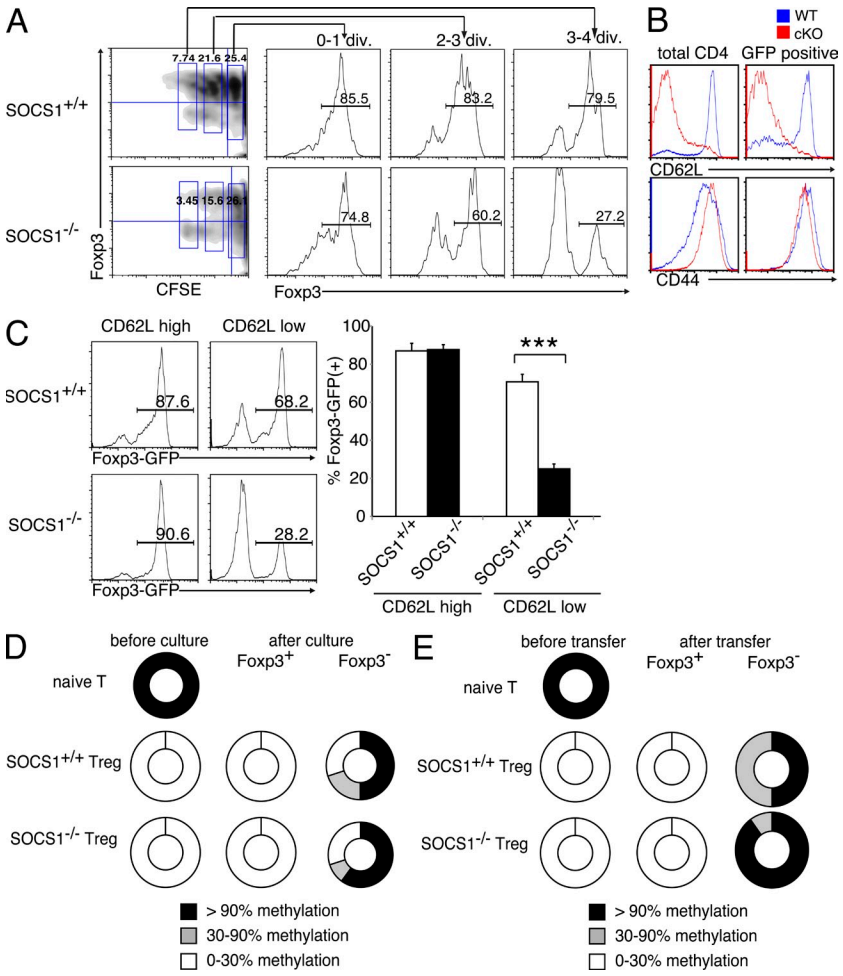

Figure 3. Foxp3 loss in SOCS1-deficient T_{reg} cells is associated with cell division and methylation of the CNS2 region. (A) Flow cytometric analysis of Foxp3 expression in *Socs1*$^{+/+}$ and *Socs1*$^{-/-}$ T_{reg} cells cultured for 3 d. Numbers on flow plots indicate the percentage of divided cells in each group (left). Each fraction was analyzed for Foxp3 staining (right). (B) Flow cytometric analysis of CD62L and CD44 expression on CD3$^+$CD4$^+$ T cells (left) and CD3$^+$CD4$^+$GFP$^+$ T cells (right) from the LN of cKO mice and WT littermate controls. (C) Flow cytometric analysis of Foxp3-GFP expression on CD62L^{high} and CD62L^{low} CD3$^+$CD4$^+$ T cells after 3-d in vitro culture (percentage of Foxp3-GFP$^+$ indicated in bar graph). Data are representative of three independent experiments (***, $P < 0.0001$). Values represent the mean ± SD. (D and E) Bisulfite sequencing of genomic DNA from WT naive T cells and T_{reg} cells from the LN of WT-*Foxp3*^{GFP} and cKO-*Foxp3*^{GFP} male mice. (D) Methylation status of the CNS2 region. After Foxp3$^+$ T_{reg} cells were cultured in vitro for 3 d, Foxp3$^+$ and Foxp3$^-$ cells were sorted. 10 CpG methylation sites in the CNS2 region of the Foxp3 gene were analyzed before culture (left) and after 3-d in vitro culture (middle and right). Foxp3-maintained cells (Foxp3$^{+\rightarrow+}$; middle) and Foxp3-lost cells (Foxp3$^{+\rightarrow-}$; right) are shown. (E) Methylation status of the CNS2 region 4 wk after transfer of Foxp3$^+$ T_{reg} cells into *Rag2*$^{-/-}$ mice. Before transfer (left), Foxp3-maintained cells (Foxp3$^{+\rightarrow+}$; middle), and Foxp3-lost cells (Foxp3$^{+\rightarrow-}$; right) are shown. Percentages of clones displaying each methylation status of CpG methylation sites are indicated in sector graphs. 10–15 clones were sequenced from each subset.

or partially methylated, and more methylation occurred in the SOCS1-defcient exFoxp3 cells than in the SOCS1-sufficient exFoxp3 cells (Fig. 3 D), which is consistent with a more rapid loss of Foxp3 in *Socs1*$^{-/-}$ T_{reg} cells than in WT T_{reg} cells.

In vivo, we also found hypermethylation of the CNS2 region in Foxp3$^-$ cells after transfer of Foxp3$^+$ cells into *Rag2*$^{-/-}$ mice (Fig. 3 E). More intensive hypermethylation in the CNS2 region in Foxp3$^-$ cells was observed in *Socs1*$^{-/-}$ T_{reg} cell transfer than in WT T_{reg} cell transfer (Fig. 3 E; >90% methylated regions were 90% in *Socs1*$^{-/-}$ vs. 50% in *Socs1*$^{+/+}$). Thus, the CNS2 region of the *Foxp3* gene in exFoxp3 cells from SOCS1-deficient T_{reg} cells was more easily hypermethylated not only in vitro but also in vivo than that from WT T_{reg} cells (Fig. 3 E).

Accelerated conversion of SOCS1-deficient T_{reg} cells into Th1- and Th17-like cells in vitro

It has been reported that T_{reg} cells convert into Th1- or Th17-like cells in appropriate in vitro culture conditions (Yang et al., 2008). To investigate the mechanism of the loss of Foxp3 and enhanced IFN-γ production from *Socs1*$^{-/-}$ T_{reg} cells in vivo, we performed an in vitro conversion assay. As shown in Fig. 4 A, when Foxp3$^+$ cells were cultured in the presence of TCR stimulation and IL-2, much higher amounts of IFN-γ as well as of IL-17 were produced from *Socs1*$^{-/-}$ T_{reg} cells than from WT T_{reg} cells. IL-12 further up-regulated IFN-γ production, whereas IL-17 was up-regulated by IL-1 and IL-6 (Fig. 4 A). Interestingly, the production of IL-17 by IL-1 and IL-6 was dependent on TGF-β because anti–TGF-β antibody abrogated the effect of IL-1 and IL-6.

Because SOCS1 has been shown to be an essential negative regulator for the JAK–STAT pathway, the activation status of STAT1 and STAT3 were compared using immunoblotting and intracellular staining (Fig. 4 B). STAT5 was reported to be phosphorylated in SOCS1-deficient T_{reg} cells at higher levels than in WT T_{reg} cells, which is associated with the increase in the number of T_{reg} cells (Lu et al., 2009). Much stronger tyrosine phosphorylation of both STAT1 and STAT3 were observed in freshly isolated SOCS1-deficient T_{reg} cells than in WT T_{reg} cells by both methods (Fig. 4 B). These data indicate that SOCS1 is necessary for the suppression of spontaneous activation of STAT1 and STAT3 in T_{reg} cells.

Foxp3 was stabilized in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells

Because SOCS1 is a strong inhibitor for the IFN-γ–STAT1 pathway, we hypothesized that hyper-STAT1 activation led to

abnormalities in SOCS1-deficient T_{reg} cells. To address this hypothesis, we used T_{reg} cells from *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice.

Most of the *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells ($CD4^+CD25^{bright}$ fraction) were $CD62L^{high}$ and $CD44^{high}$, suggesting that IFN-γ deficiency diminished $CD62L^{low}$ T_{reg} cell subsets (Fig. 5 A). Consistently, when T_{reg} cells from both *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice were transferred into *Rag2*$^{-/-}$ mice, colitis did not develop even 6 wk after transfer (Fig. 5 B). Foxp3 expression in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells was as stable as in *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ T_{reg} cells and WT T_{reg} cells after transfer into *Rag2*$^{-/-}$ mice (Figs. 2 D and 5 C). After transfer, unlike in *Socs1*$^{-/-}$ T_{reg} cells, CD25 and CTLA4 were maintained at significantly higher levels in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells (Fig. 5 D, top and bottom). We examined the methylation status of the CNS2 region in both *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells in vitro. The CNS2 region of T_{reg} cells from the LN of *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice were fully demethylated (Fig. 5 E). After 3-d culture, the Foxp3-maintaining fraction (Foxp3$^{+\rightarrow+}$) retained full demethylation in the CNS2 region in both *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells. Methylation levels of the CNS2 region in the *Ifnγ*$^{-/-}$ exFoxp3 fraction (Foxp3$^{+\rightarrow-}$) were lower than those in the *Ifnγ*$^{+/+}$ exFoxp3 fraction (Figs. 3 D and 5 E), suggesting that IFN-γ played a positive role in the methylation of the CNS2 region in T_{reg} cells. The rate of methylation in the CNS2 region in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ exFoxp3 cells was lower than that in *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ exFoxp3 cells. These results were consistent with a stable Foxp3 expression in *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells after transfer into *Rag2*$^{-/-}$ mice.

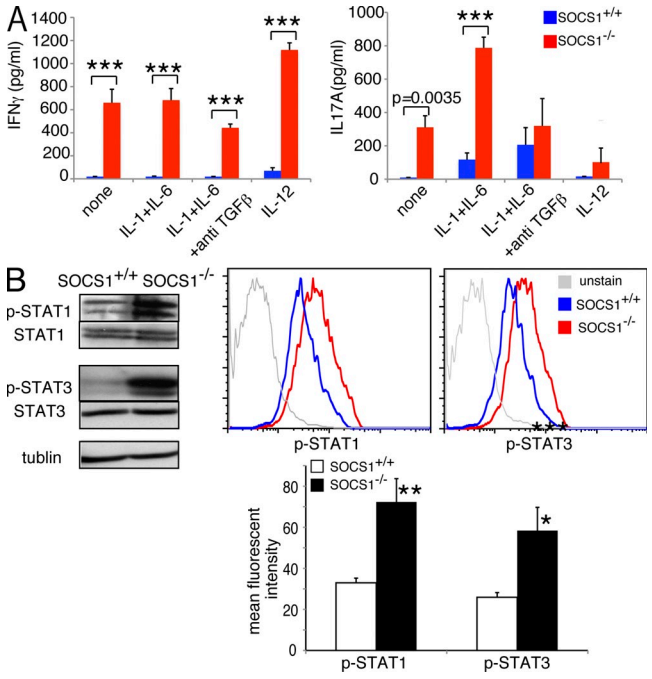

Figure 4. SOCS1-deficient T_{reg} cells convert into Th1- and Th17-like cells in vitro. (A) 10^5 *Socs1*$^{+/+}$ and *Socs1*$^{-/-}$ T_{reg} cells/well were activated with anti-CD3/anti-CD28 beads, 10 ng/ml IL-2, and the indicated cytokines, and IFN-γ and IL-17A were measured by ELISA (***, $P < 0.0001$). (B) Detection of phosphorylations of STAT1 and STAT3 in T_{reg} cells with the indicated genotypes by immunoblot (left) and flow cytometry (right; mean fluorescent intensity is indicated in the bar graph; *, $P < 0.05$; **, $P < 0.01$). (A and B) Data are representative of five independent experiments. Values represent the mean ± SD.

Ifnγ$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells still lacked in vivo suppression activity because of stronger Th17 instruction

To test the suppression activity, T_{reg} cells from *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice were then transferred into *Rag2*$^{-/-}$ mice with naive T cells. 4 wk after transfer, T_{reg} cells from *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ mice suppressed colitis elicited by naive T cells (Fig. 6 A and Fig. S7). The suppression activity of *Ifnγ*$^{-/-}$ T_{reg} cells was lower than that of *Ifnγ*$^{+/+}$ T_{reg} cells (Figs. 2 A and 6 A), yet recipient mice did not develop severe colitis 4 wk after T cell transfer. To our surprise, *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells could not prevent colitis, which was confirmed by body weight loss and histological examination (Fig. 6 A and Fig. S5). Expression of Foxp3 in *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ T_{reg} cells as well as in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells was maintained in recipient *Rag2*$^{-/-}$ mice (Fig. 6 B), just as it was in the transfer of T_{reg} cells alone (Fig. 2 B). Therefore, Foxp3 maintenance is not sufficient for the suppression of effector T cell functions in vivo.

To understand these unexpected results, we examined the production of inflammatory cytokines from T_{reg} cells or naive/effector cells transferred into *Rag2*$^{-/-}$ mice. IL-10 levels were not significantly different between *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells (Fig. S4). Because Th17 has also been implicated in colitis (Chaudhry et al., 2009; Durant et al., 2010), we then compared IFN-γ and IL-17 production from naive/effector ($Ly5.1^+$) T cells (Fig. 6 C, top) and T_{reg} cells ($Ly5.1^-$, $Ly5.2^+$; Fig. 6 C, middle) in the LN of transferred recipient mice, after which percent positivity values of cytokine production were determined by flow cytometry (Fig. 6 C, bottom). As shown in Fig. 6 C, when naive T cells alone were transferred into *Rag2*$^{-/-}$ mice, a portion of these cells converted into effector cells that produced mainly IFN-γ, but with very low levels of IL-17 (Fig. 6 C, a). When naive T cells and WT T_{reg} cells were cotransferred, differentiation of Th1-like cells from naive T cells was completely suppressed (Fig. 6 C, b). However, when naive T cells and *Socs1*$^{-/-}$ T_{reg} cells were cotransferred, both IFN-γ– and IL-17–producing cells appeared from transferred naive T cells, which is consistent with the failure of suppression of colitis by *Socs1*$^{-/-}$ T_{reg} cells (Fig. 6 C, c). Thus, SOCS1-deficient T_{reg} cells promoted Th1 and Th17 cell differentiation from naive T cells, whereas WT T_{reg} cells did not. In contrast, when naive T cells and *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ T_{reg} cells were cotransferred, a significant fraction of IL-17–producing cells appeared from transferred naive T cells (Fig. 6 C, d). IL-17 was also produced from transferred T_{reg} cells. These results could explain why *Ifnγ*$^{-/-}$ T_{reg} cells did not prevent colitis very efficiently (Fig. 6 A). Much more intense IL-17 production was observed from both naive T cells and T_{reg} cell fractions in recipient mice cotransferred with naive

T cells and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells (Fig. 6 C, e). These data suggest that the cytokine profile of T_{reg} cells instructs the direction of differentiation of Th1 or Th17 from naive T cells in lymphopenic conditions.

Instruction of Th1 and Th17 cell differentiation by cytokines from T_{reg} cells through APCs in vitro

To confirm these instructions by T_{reg} cells, we first measured IL-17 from IFN-γ–deficient T_{reg} cells in in vitro culture experiments. As expected, *Ifnγ*$^{-/-}$ T_{reg} cells produced higher amounts of IL-17 than did WT T_{reg} cells, and IL-17 production was further enhanced by SOCS1 deficiency (Fig. 7 A). These data suggest that IFN-γ usually suppressed IL-17 production from T_{reg} cells, which skewed Th1 cell differentiation from naive T cells. Coincidently, hyper-STAT1 activation was not observed in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells, although STAT3 activation was still high (Fig. 7 B, top and bottom). Thus, hyper STAT3 activation could account for high levels of IL-17 production from *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells.

The effect of culture supernatants of T_{reg} cells on Th1/Th17 cell differentiation was then examined (Fig. 7 C). Culture supernatants of T_{reg} cells were added to the co-culture of naive T cells only or in the presence or absence of APCs (Fig. 7 C). In the absence of APCs, culture supernatants from *Socs1*$^{+/+}$ T_{reg} cells, *Socs1*$^{-/-}$ T_{reg} cells, or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells did not promote Th1 or Th17 cell differentiation, suggesting that cytokines from T_{reg} cells modify the APCs for accelerated Th1 or Th17 cell differentiation form naive T cells (Fig. 7 C, top). Supernatants from *Socs1*$^{-/-}$ T_{reg} cells instructed naive T cells to differentiate into IL-17–producing effector cells when cultured with LPS-untreated APCs (Fig. 7 C, middle; and Fig. S6, right). This finding is consistent with our observations that *Socs1*$^{-/-}$ T_{reg} cells produced IL-17 (Fig. 4 A) and induced more Th17 in vivo (Fig. 6 C). This instruction of naive T cells was augmented by *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells, which produced the largest amount of IL-17 (Fig. 7 A), consistent with the results shown in Fig. 6 C. When APCs were activated by LPS (Fig. 7 C, bottom; and Fig. S6, left), naive T cells, after the addition of supernatant of *Socs1*$^{-/-}$ T_{reg} cells, were instructed to differentiate into IFN-γ–producing effector cells, which is again consistent with higher IFN-γ production from *Socs1*$^{-/-}$ T_{reg} cells (Fig. 4 A), and induced more Th1 in vivo (Fig. 6 C). *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells did not induce Th1 in LPS-treated APCs, suggesting that IFN-γ production from T_{reg} cells plays an important role in the Th1 instruction of naive T cells. Both IFN-γ and IL-17 antibodies abolished Th1 and Th17 instruction, respectively, confirming that IFN-γ and IL-17 from SOCS1-deficeint T_{reg} cells play essential roles in Th1 and Th17 induction from naive T cells (Fig. S6).

To confirm the role of APCs, we examined the effect of anticytokine antibodies on the in vitro instruction experiments (Fig. S6). Th1 instruction was eliminated by IL-12 antibodies, suggesting that IL-12 from APCs promoted Th1 cell differentiation. Consistent with this

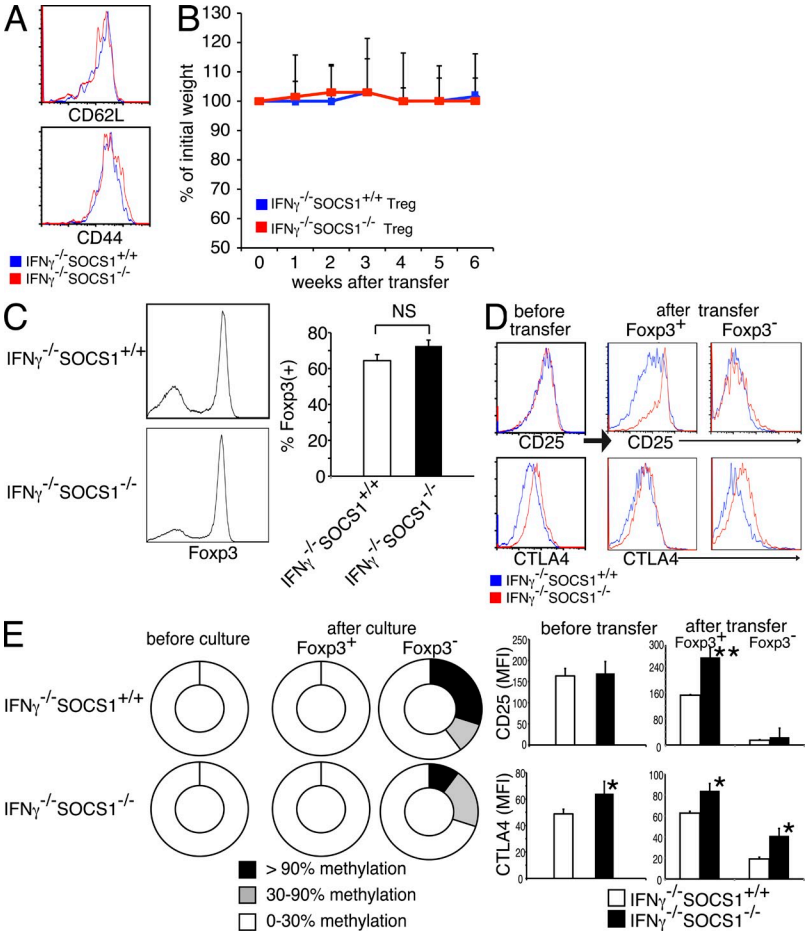

Figure 5. IFN-γ suppresses CNS2 methylation and Foxp3 loss in SOCS1-deficient T_{reg} cells. (A) Expression profiles of CD62L and CD44 on CD3$^+$CD4$^+$CD25^{bright} cells of the LN cells from *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice. (B–D) 2 × 10^5 T_{reg} cells from *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ mice or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice were transferred into *Rag2*$^{-/-}$ mice. 6 wk after transfer, recipient mice were analyzed. (B) Body weight changes. (C) Flow cytometric analysis of Foxp3 expression on CD3$^+$CD4$^+$ T cells from the LN in *Rag2*$^{-/-}$ mice (percentage of Foxp3$^+$ indicated in bar graph). (D) CD25 and intracellular CTLA4 expression on CD3$^+$CD4$^+$Foxp3$^+$ cells and Foxp3$^-$ cells from the LN in *Rag2*$^{-/-}$ mice (right) compared with those on CD3$^+$CD4$^+$CD25^{bright} cells before transfer (left; mean fluorescent intensity [MFI] indicated in bar graph). Data are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.01$). Values represent the mean ± SD. (E) Bisulfite sequencing of genomic DNA from T_{reg} cells from the LN of *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ male mice. 10 CpG methylation sites in the CNS2 region of the Foxp3 gene were analyzed before culture (left) and after 3-d in vitro culture. Percentages of clones displaying each methylation status of CpG methylation sites are indicated in sector graphs. 10–15 clones were sequenced from each subset.

notion, *Socs1*$^{-/-}$ T_{reg} cells could not suppress colitis induced by *Ifnγr1*$^{-/-}$ naive T cells transferred into *Rag2*$^{-/-}$ mice (Fig. S7). This experiment suggested that IFN-γ from *Socs1*$^{-/-}$ T_{reg} cells still induced colitogenic T cells from *Ifnγr1*$^{-/-}$ naive T cells. In contrast, anti–IL-6 antibody completely inhibited and anti–TGF-β antibody partially inhibited Th17 instruction. Anti–IL-23 antibody did not affect Th17 cell differentiation. These results suggest that IL-17 from cultured T_{reg} cells stimulated IL-6 and TGF-β production from APCs, which promoted Th17 cell differentiation from naive T cells.

To confirm this possibility, we used IL-17–deficient naive T cells for the transfer. As shown in Fig. 7 D, *Rag2*$^{-/-}$ mice transferred with IL-17–deficient naive T cells developed colitis, which was less severe than in WT naive T cell transfer. However, *Socs1*$^{-/-}$ T_{reg} cells caused extremely severe colitis (Fig. 7 D), which was triggered by the large levels of IFN-γ (not depicted). As we expected, *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells as well as *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ T_{reg} cells suppressed colitis induced by IL-17–deficient naive T cell transfer (Fig. 7 D). These data further support our hypothesis that *Ifnγ*$^{-/-}$ T_{reg} cells instruct Th17 cell differentiation of naive T cells, thereby suppressing colitis less efficiently, and these processes are accelerated when SOCS1 is deficient in *Ifnγ*$^{-/-}$ T_{reg} cells.

DISCUSSION

In this study, we demonstrated that SOCS1 is necessary for T_{reg} stability and suppressor functions: SOCS1 protects T_{reg} cells from harmful effects of inflammatory cytokines, which promote the loss of Foxp3 expression and the conversion into Th1/Th17-like effector cells. Recent studies have shown that T_{reg} cells rapidly lose Foxp3 expression upon the transfer into a lymphopenic host (Komatsu et al., 2009) or in inflammatory conditions (Zhou et al., 2009). Such exFoxp3 cells (Zhou et al., 2009) or lapsed T_{reg} cells (Murai et al., 2010) develop an effector-memory phenotype, produce pathogenic cytokines, and can trigger the development of autoimmunity. Multiple studies have suggested that T_{reg} cells isolated from inflammatory sites express reduced amounts of Foxp3, possibly increasing susceptibility to autoimmunity (Wan and Flavell, 2007; Tang et al., 2008).

Foxp3 expression is regulated by various factors, such as Smad2/3 (Takimoto et al., 2010), Runx1 (Kitoh et al., 2009; Rudra et al., 2009), STAT5 (Yao et al., 2007), c-Rel (Hori, 2010), and DNA methylation of the CNS2 region in the Foxp3 enhancer (Zheng et al., 2010). DNA methylation of CNS2 is particularly important because CNS2 is required for Foxp3 expression in the progeny of dividing T_{reg} cells (Zheng et al., 2010). Surprisingly, we found that the CNS2 region of freshly isolated SOCS1-deficient T_{reg} cells was fully demethylated, indicating that they are committed Foxp3$^+$ T_{reg} cells. In *Rag2*$^{-/-}$ mice as well as in in vitro culture, part of the

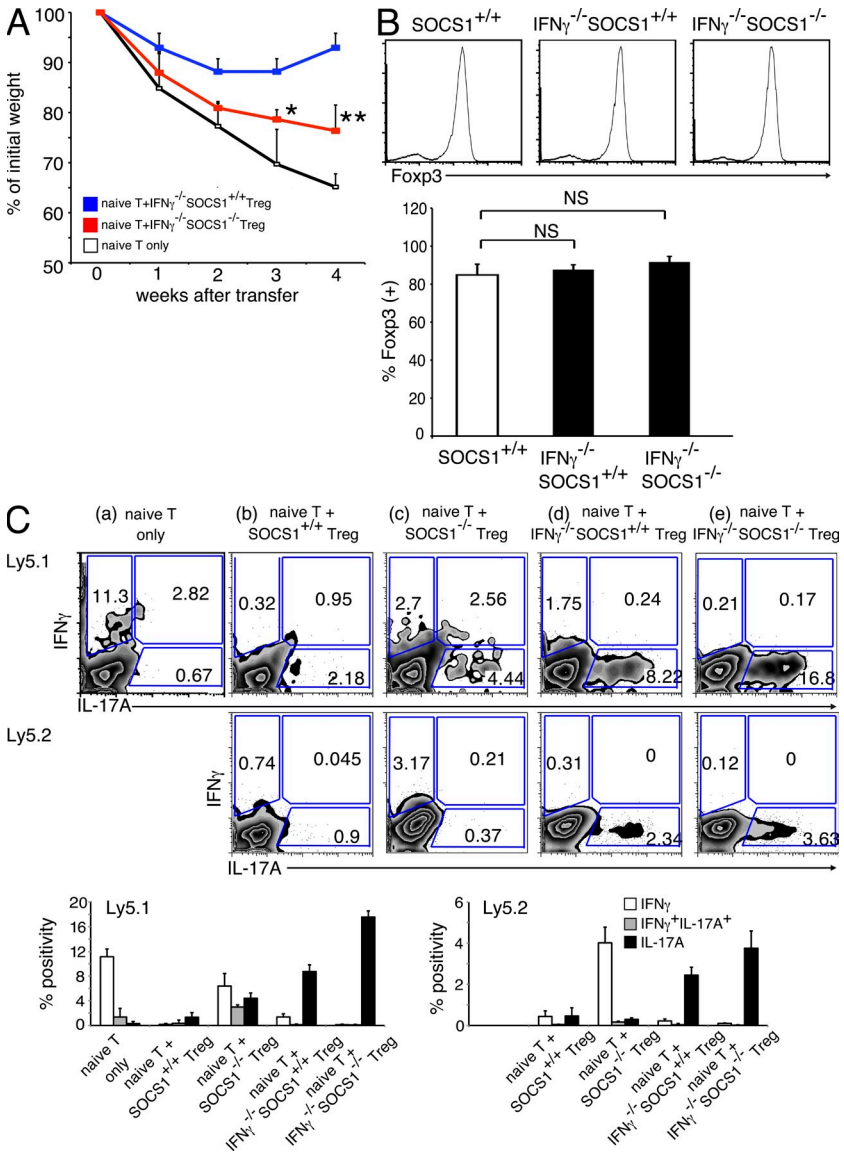

Figure 6. *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells fail to suppress colitis in vivo. (A and B) 2 × 10^5 Ly5.2 *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ or Ly5.2 *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells were cotransferred with 4 × 10^5 Ly5.1 naive T cells into *Rag2*$^{-/-}$ mice. 4 wk after transfer, recipient *Rag2*$^{-/-}$ mice were analyzed. (A) Body weight changes. (B) Flow cytometric analysis of Foxp3 expression on Ly5.2$^+$CD3$^+$CD4$^+$ T cells from the LN in *Rag2*$^{-/-}$ mice (percentage of Foxp3$^+$ indicated in bar graph). (C) Flow cytometric analysis of IFN-γ and IL-17A on Ly5.1$^+$CD3$^+$CD4$^+$cells (top) and Ly5.2$^+$CD3$^+$CD4$^+$ T cells (middle) from the LN in recipient mice (percent positivity, bottom). (A–C) Data are representative of three independent experiments (*, P < 0.05; **, P < 0.01). Values represent the mean ± SD.

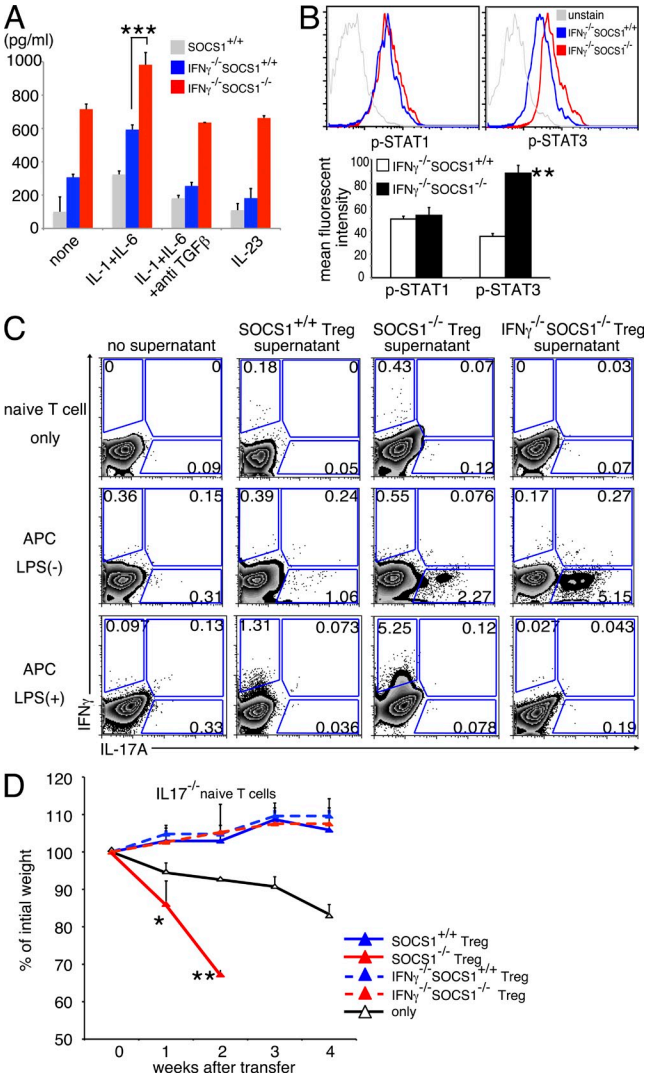

Figure 7. exFoxp3 cells instruct differentiation of naive T cells to Th1 and Th17 in vitro. (A) 10^5 *Socs1*$^{+/+}$, *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$, or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells/well were stimulated with anti-CD3/anti-CD28 beads, 10 ng/ml IL-2, and the indicated cytokines, and IL-17A was measured by ELISA. Data are representative of five independent experiments (***, $P < 0.001$). (B) Phosphorylation status of STAT1 and STAT3 in T_{reg} cells with the indicated genotypes by flow cytometry (mean fluorescent intensity shown in bar graph). Data are representative of five independent experiments (**, $P < 0.01$). (C) 3×10^5 WT naive T cells/well were cultured for 5 d alone (top) or with untreated APCs (middle) or LPS-stimulated APCs (bottom ; 7×10^5 T cell–depleted spleen cells/well) plus supernatants from *Socs1*$^{+/+}$, *Socs1*$^{-/-}$, or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells (2×10^5/well) cultured for 2 d in the presence of anti-CD3/anti-CD28 beads and 10 ng/ml IL-2. Flow cytometric analysis of IFN-γ and IL-17A on these cells is shown. Data are representative of three independent experiments. (D) 2×10^5 *Socs1*$^{+/+}$, *Socs1*$^{-/-}$, *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$, or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells were cotransferred with 4×10^5 *Il17*$^{-/-}$ naive T cells into *Rag2*$^{-/-}$ mice, and body weight is shown. Data are representative of three independent experiments (*, $P < 0.05$; **, $P < 0.01$). (A, B, and D) Values represent the mean ± SD.

committed Foxp3$^+$ cells expanded and converted into Foxp3$^-$ cells (i.e., exFoxp3 cells). The CNS2 region in SOCS1-deficient exFoxp3 cells was more methylated than that in WT T_{reg} cells. Also, because the CNS2 methylation status of T_{reg} cells was altered after 3-d in vitro culture and there was a partially methylated CNS2 region, it is unlikely that all of the exFoxp3 cells were caused by the expansion of contaminated Foxp3$^-$ effector T cells. Accordingly, we concluded that SOCS1 contributes to T_{reg} cell integrity by maintaining stable Foxp3 expression.

Our present study indicated that the excessive IFN-γ–STAT1 signals resulted in faster loss of Foxp3 expression in T_{reg} cells in lymphopenic conditions as well as in an in vitro culture system. One possibility is that the IFN-γ–STAT1 pathway increases reprogramming from SOCS1-deficient T_{reg} cells to effector cells. IFN-γ may promote the expansion or development of memory type CD62L^{low} cells because we observed that the CD62L^{low} fraction was at normal levels in IFN-γ/SOCS1 double-deficient T_{reg} cells. STAT1 may antagonize STAT5, yet this is unlikely because we could not observe the reduction of STAT5 phosphorylation in our SOCS1-deficient T_{reg} cells. STAT1 has also been shown to inhibit the TGF-β–Smad pathway (Tanaka et al., 2008). The phenotypes of Smad2/3-deficient T_{reg} cells were similar to those observed in SOCS1-deficient T_{reg} cells (Takimoto et al., 2010). Thus, the suppression of Smad signaling by STAT1 could be one of the mechanisms behind the reprogramming of T_{reg} cells. The relationship between these factors and the reprogramming of T_{reg} cells remain to be clarified.

Recently, Rubtsov et al. (2010) reported that highly purified T_{reg} cells were very stable under physiological and inflammatory conditions. Lu et al. (2010) reported that Foxp3 expression of SOCS1-deficient T_{reg} cells from *Foxp3*Cre-*Socs1*$^{f/f}$ mice was maintained in the presence of naive and effector T cells, although the suppression activity of *Socs1*$^{-/-}$ T_{reg} cells was defective and IFN-γ was produced from *Socs1*$^{-/-}$ T_{reg} cells with STAT1 hyperactivation. Thus, Foxp3 expression in *Socs1*$^{-/-}$ T_{reg} cells from *Foxp3*Cre-*Socs1*$^{f/f}$ mice was more stable than that in *Socs1*$^{-/-}$ T_{reg} cells from *Lck*Cre-*Socs1*$^{f/f}$ mice. Because SOCS1 of *Lck*Cre-*Socs1*$^{f/f}$ mice is deleted in the T_{reg} cell progenitors, whereas SOCS1 of *Foxp3*Cre-*Socs1*$^{f/f}$ mice is deleted after T_{reg} cell maturation, this discrepancy may be explained by differences in the developmental conditions of T_{reg} cells. It is also possible that in *Lck*Cre-*Socs1*$^{f/f}$ mice, cytokines from *Socs1*$^{-/-}$ effector T cells could contribute to more drastic phenotypic changes in *Socs1*$^{-/-}$ T_{reg} cells.

A recent study has demonstrated that during lethal *Toxoplasma gondii* infection, the exposure of WT T_{reg} cells to high amounts of Th1 inflammatory mediators superimposes a Th1 effector program on T_{reg} cells of not only the Foxp3-losing portion but also the Foxp3-maintaining portion (Oldenhove et al., 2009). Thus, the conversion of T_{reg} cells to Th1 is one of the mechanisms for uncontrolled Th1 response in STAT1-hyperactivated T_{reg} cells, and this process is usually protected by SOCS1. Recently, Lu et al. (2010) reported on the defective suppression activity of SOCS1-deficient T_{reg} cells and IFN-γ production from these T_{reg} cells by STAT1 hyperactivation. In addition, our data suggest the intriguing possibility that

hyperactivation of STAT3 in T_{reg} cells facilitates the conversion to Th17-like cells, which leads to an uncontrolled response in effector Th17 cells. STAT5, in contrast, enhances Foxp3 expression and the number of T_{reg} cells (Lu et al., 2009).

Like STAT1, STAT3 has been reported to inhibit Foxp3 expression during iT_{reg}/Th17 cell differentiation together with TGF-β (Takimoto et al., 2010). However, the role of STAT3 in Foxp3 stability in T_{reg} cells seems to be more complicated. STAT3-deficient T_{reg} cells have been shown to expand less efficiently than WT T_{reg} cells in lymphopenic conditions (Durant et al., 2010) and lose their suppression activity, especially for Th17 (Chaudhry et al., 2009; Durant et al., 2010). Foxp3 was stable in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells with hyperactivated STAT3. Thus, it is unlikely that the hyperactivation of STAT3 is linked to Foxp3 instability. Previously, we have shown that STAT3 activation is reduced in SOCS1-deficient naive T cells in response to IL-6 and TGF-β caused by SOCS3 induction (Tanaka et al., 2008). The mechanism of hyperactivation of STAT3 in SOCS1-deficient T_{reg} cells remains to be investigated. Although Foxp3 expression of *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells is stabilized, these *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells were still defective in terms of the suppression of IL-17 production from both T_{reg} cells and effector T cells in *Rag2*$^{-/-}$ mice. This indicates that the stable expression of Foxp3 is not a sufficient condition for the suppression of effector Th17 cells. This may be linked to a high conversion potential of T_{reg} cells to Th17 cells. In a recent study using IL-17–RFP reporter mice with a Foxp3-GFP reporter, Yang et al. (2008) demonstrated the presence of a RFP$^+$GFP$^+$ transient phase upon T cell activation in vitro and in vivo. Thus, it is highly possible that both Th17 and T_{reg} cell programs could occur simultaneously in one cell, which underlies the conversion of Foxp3-positive T_{reg} cells to IL-17–producing cells. We observed that STAT3 was hyperactivated in *Socs1*$^{-/-}$ T_{reg} cells as well as in *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells. Although we do not have direct proof demonstrating that STAT3 hyperactivation is responsible for the conversion of T_{reg} cells to Th17, it is likely true because IL-17 production from nT_{reg} cells was enhanced by IL-6 and IL-1 and because STAT3, in addition to TGF-β signals, has been shown to be an essential factor for the generation of Th17.

SOCS1-deficient T_{reg} cells, which convert to Th1- and Th17-like cells, seem to instruct naive T cells to differentiate into the same cytokine-producing effector T cells. This can also explain why STAT1 or STAT3 hyperactivated T_{reg} cells cannot control Th1 or Th17 effectors, respectively. Intriguingly, previous studies have indicated that STAT1–T-bet (Koch et al., 2009) and STAT3 (Chaudhry et al., 2009; Durant et al., 2010) in T_{reg} cells are required for Th1 and Th17 suppression, respectively. Our current study, as well as a previous study (Lu et al., 2010), clearly indicates that both the lack of STAT1/3 and unrestrained STAT1/3 activation in T_{reg} cells leads to a severe failure of immunological tolerance.

Anti–IFN-γ, –IL-17, –IL-12, and –IL-6 antibodies nearly completely inhibited Th1 and Th17 cell differentiation (Fig. S6). Thus, we clarified that IFN-γ from Th1-converted T_{reg} cells and IL-17 from Th17-converted T_{reg} cells preferentially induced effector Th1 and Th17 cell differentiation, respectively, by modulating APCs. It has been reported that T_{reg} cells interact with DCs before naive T cells and down-regulate the expression of CD80/86 caused by high levels of CTLA4 on T_{reg} cells (Onishi et al., 2008). Because SOCS1-deficient T_{reg} cells lose CTLA4 expression, they may not be able to suppress the expression levels of co-stimulator ligands. It has been reported that DCs treated with IFN-γ preferentially induce Th1 (Hanada et al., 2003), whereas IL-17–treated DCs preferentially induce Th17 cell differentiation because of the higher production of IL-1β, IL-6, and IL-23 from IL-17–treated DCs (Sutton et al., 2009). IL-17 may not directly induce Th17 cell differentiation in the absence of APCs because IL-17 has been reported to actually reduce Th17 cell differentiation (Smith et al., 2008). These previous studies support our results, and IFN-γ and IL-17 from T_{reg} cells are thought to instruct effector T cell differentiation into Th1 or Th17 by modulating APCs.

In conclusion, we propose the idea that SOCS1 is an important guardian of T_{reg} cells. Our findings also raise the interesting possibility that up-regulation of SOCS1 in T_{reg} cells at appropriate levels reinforces T_{reg} cell functions because SOCS1 may protect T_{reg} cells from harmful effects of inflammatory cytokines that accelerate the conversion of T_{reg} cells into effectors. These findings may improve T_{reg} cell therapy for autoimmune diseases and organ transplantations.

MATERIALS AND METHODS

Mice. *Lck*Cre-*Socs1*$^{f/f}$ (T cell–specific *Socs1*-cKO; Tanaka et al., 2008) and *Socs1*$^{f/f}$ (littermate control; WT) mice (sex and age matched) were used. cKO mice and *Socs1*$^{f/f}$ littermates crossed with knockin mice with Foxp3-IRES-GFP (WT-*Foxp3*^{GFP} mice and cKO-*Foxp3*^{GFP} mice) were provided by B. Malissen (Université de la Méditerranée, Marseille, France; Wang et al., 2008). *Ifnγ*$^{-/-}$, *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$, *Il17*$^{-/-}$, and *Ifnγr1*$^{-/-}$ mice with the C57BL/6J background were described previously (Nakae et al., 2002; Hanada et al., 2003; Suzue et al., 2003). Mice were kept in conventional conditions in Keio University. All experiments using these mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Keio University.

Histopathologic examination. Tissue samples were obtained from the proximal and distal colon, skin, liver, and kidneys and then fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Assessments of the severity of colitis were performed using the histopathologic score as described previously (Inagaki-Ohara et al., 2004).

In vivo suppression assay and transfer colitis model in *Rag2*$^{-/-}$ mice. Flow cytometry–purified 4×10^5 CD4$^+$CD25$^-$CD62L$^+$CD44$^-$ naive T cells from WT mice were injected intravenously into *Rag2*$^{-/-}$ mice in combination with 2×10^5 CD3$^+$CD4$^+$CD25$^+$Foxp3^{GFP} cells from WT-*Foxp3*^{GFP} or cKO-*Foxp3*^{GFP} mice (8 wk old and sex matched) or 2×10^5 CD3$^+$CD4$^+$CD25^{bright} T_{reg} cells from *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice (8 wk old and sex matched). Mice were observed daily and weighed weekly. 4 wk after cell transfer, the mice were sacrificed for experiments, and colon sections were stained with H&E. Tissues were graded semiquantitatively. Histological grades were assigned in a blinded manner.

Transfer of T_{reg} cells into *Rag2*$^{-/-}$ mice. Flow cytometry–purified 2×10^5 CD3$^+$CD4$^+$CD25$^+$Foxp3^{GFP} cells from WT-*Foxp3*^{GFP} or cKO-*Foxp3*^{GFP} mice (8 wk old and sex matched) or 2×10^5 CD3$^+$CD4$^+$CD25^{bright} T_{reg} cells from *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$ or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice (8 wk old and sex matched) were injected intravenously into *Rag2*$^{-/-}$ mice. Mice were observed daily and weighed weekly. 6 wk after cell transfer, the mice were sacrificed for experiments.

Flow cytometry, cell sorting, and cytokine secretion assays. Cell surface staining and flow cytometric analysis of CD3, CD4, CD25, CD62L, and CD44 (all from eBioscience) expression were performed as described previously (Fontenot et al., 2005). For the isolation of T_{reg} cells, $CD4^+$ T cells were positively selected with magnetic-activated cell sorting (Miltenyi Biotec), and $CD3^+CD4^+CD25^+Foxp3^{GFP}$ cells or $CD3^+CD4^+CD25^{bright}$ cells were further purified using a FACSAria cell sorter (BD). The purity of the sorted populations was invariably >99%. Intracellular staining of Foxp3, IL-2, IFN-γ, IL-17A, and CTLA4 (all from eBioscience) was performed after fixation and permeabilization according to the manufacturer's instructions. To measure T cell cytokine production, cells were stimulated with 50 ng/ml PMA and 250 ng/ml ionomycin in the presence of Golgi Plug (BD) for 4 h at 37°C before staining. For phospho-STAT1 and phospho-STAT3 staining, Phosflow Lyse/Perm buffer and Perm Buffer III (BD) were used according to the manufacturer's instructions.

In vitro culture of T_{reg} cells. To examine effector T cell differentiation, 10^5 T_{reg} cells/well ($CD3^+CD4^+CD25^+$ $Foxp3^{GFP}$ cells or $CD3^+CD4^+CD25^{bright}$ cells) from WT-*Foxp3*^{GFP}, cKO-*Foxp3*^{GFP}, *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$, or *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice (8 wk old and sex matched) were stimulated with anti-CD3/anti-CD28 mAb–coated beads (Dynal) at a 1:1 cell/bead ratio for 72 h, with 10 ng/ml IL-2 in 96-well, flat-bottom plates.

In vitro effector T cell differentiation with supernatants of cultured T_{reg} cells. 3×10^5 WT naive T cells/well were cultured for 5 d without, with LPS-untreated, or with 1 µg/ml LPS–stimulated APCs (7×10^5 T cell–depleted spleen cells/well) and supernatants from 2-d cultured 2×10^5 *Socs1*$^{+/+}$, *Socs1*$^{-/-}$, and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells/well. Flow cytometric analysis of IFN-γ and IL-17A was performed on these cells. These were also observed with 10 µg/ml anti–IFN-γ, –IL-12, –IL-17, –IL-6, –TGF-β, or –IL-23 antibodies.

Cytokine detection. Supernatants from cultures of T_{reg} cells with or without the indicated cytokines were harvested and pooled. Where indicated, 20 ng/ml IL-1α, 20 ng/ml IL-1β, 40 ng/ml IL-6, 50 ng/ml IL-12, 50 ng/ml IL-23, and 10 µg/ml anti–TGF-β mAb (1D11) were added. IFN-γ and IL-17A concentrations were measured using commercially available mouse ELISA Ready-S (eBioscience). All samples were run in triplicate.

Western blot analysis. Cell lysates from T_{reg} cells (2×10^5 cells) from WT-*Foxp3*^{GFP}, cKO-*Foxp3*^{GFP}, *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$, and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice were resolved by SDS-PAGE subjected to Western blot analysis. The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase–conjugated antibodies against rabbit or mouse IgG (Jackson ImmunoResearch Laboratories, Inc.), using Chemi-Lumi One Super Western blotting detection reagents (Nacalai Tesque). Antibodies against STAT1, phospho-STAT1, STAT3, phospho-STAT3 (all from Cell Signaling Technology), and tubulin (Sigma-Aldrich) were used to visualize the corresponding proteins.

Reverse transcription PCR analysis. Total RNA was prepared using a nucleospin RNA XS (MACHEREY-NAGEL). RNA was reverse transcribed to cDNA with random primers (Applied Biosystems) and a high capacity cDNA reverse transcription kit (Applied Biosystems) in accordance with the manufacturer's protocol. To determine the cellular expression level of each gene, quantitative real-time PCR analysis was performed using a C1000 Thermal Cycler (Bio-Rad Laboratories). The PCR mixture consisted of 5 µl of KAPA SYBR FAST qPCR kits (Kapa Biosystems), 15 pmol of forward and reverse primers, and the cDNA samples in a total volume of 10 µl. Relative RNA abundance was determined based on control-HPRT abundance.

Bisulfite sequencing. Genomic DNA extracted from freshly isolated or 3-d cultured 2×10^5 T_{reg} cells from male WT-*Foxp3*^{GFP}, cKO-*Foxp3*^{GFP}, *Ifnγ*$^{-/-}$*Socs1*$^{+/+}$, and *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ mice (8 wk old and sex matched) was digested with BamHI. 2×10^5 T_{reg} cells from male WT-*Foxp3*^{GFP} and cKO-*Foxp3*^{GFP} mice before transfer or 4 wk after transfer into *Rag2*$^{-/-}$ mice were also examined. H_2O was added to 500 ng of the digested DNA to a volume of 19 µl, and 1.2 µl of 6N NaOH was added; the mixture was then incubated at 37°C for 15 min. Next, 120 µl of 3.6N Na bisulfite, 0.57 mM hydroquinone, and 0.3N NaOH were added. Samples were then treated with 15 cycles of 95°C for 30 s to 50°C for 15 min. The reaction was then desalted using the Wizard DNA cleanup system (Promega) and eluted with 50 µl TE (Tris-EDTA). 3 µl of 5N NaOH was added, and the samples were incubated for 5 min at room temperature. The products were then ethanol precipitated and dissolved with 50 µl of TE buffer. The CNS2 region was PCR amplified with the primer set (forward, 5′-TTTTGGGTTTTTTTGGTATTTAAGA-3′; reverse, 5′-TTAACCAAATTTTTCTACCATTAAC-3′) and T/A cloned into a pGEM-T-Easy vector (Promega). 10 inserted plasmids from each condition were purified and sequenced. 2×10^5 T_{reg} cells before transfer and 4 wk after transfer into *Rag2*$^{-/-}$ mice were also analyzed.

Statistical analysis. For statistical analysis, we used the Student's *t* test.

Online supplemental material. Fig. S1 shows that the suppressive activity of SOCS1-deficient T_{reg} cells is attenuated in *Rag2*$^{-/-}$ mice. Fig. S2 shows colitis caused by SOCS1-deficient T_{reg} cells in *Rag2*$^{-/-}$ mice. Fig. S3 shows that the loss of Foxp3 expression in T_{reg} cells is not caused by outgrowth of activated memory T cell contamination of injected donor cells in *Rag2*$^{-/-}$ mice. Fig. S4 shows expression levels of IL-10 of T_{reg} cells before and after transfer in *Rag2*$^{-/-}$ mice. Fig. S5 shows that the suppressive activity of *Ifnγ*$^{-/-}$*Socs1*$^{-/-}$ T_{reg} cells is attenuated in *Rag2*$^{-/-}$ mice. Fig. S6 shows that anti–IFN-γ, –IL-17, –IL-12, and –IL-6 antibodies nearly completely inhibit Th1 and Th17 cell differentiation through APCs. Fig. S7 shows that *Socs1*$^{-/-}$ T_{reg} cells cannot suppress colitis induced by the transfer of *Ifnγr1*$^{-/-}$ naive T cells into *Rag2*$^{-/-}$ mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110428/DC1.

We thank N. Shiino for providing technical assistance and N. Aizawa for manuscript preparation.

This work was supported by special grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, the Takeda Science Foundation, the Uehara Memorial Foundation, the Mitsubishi Pharma Research Foundation, the Kanae Foundation for the Promotion of Medical Science, the Mochida Memorial Foundation, and the Program for the Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation (to A. Yoshimura).

The authors have no conflicting financial interests.

Submitted: 24 February 2011
Accepted: 8 August 2011

REFERENCES

Belkaid, Y., and K. Tarbell. 2009. Regulatory T cells in the control of host-microorganism interactions. *Annu. Rev. Immunol.* 27:551–589. http://dx.doi.org/10.1146/annurev.immunol.021908.132723

Bennett, C.L., and H.D. Ochs. 2001. IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena. *Curr. Opin. Pediatr.* 13:533–538. http://dx.doi.org/10.1097/00008480-200112000-00007

Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27:20–21. http://dx.doi.org/10.1038/83713

Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27:68–73. http://dx.doi.org/10.1038/83784

Chan, H.C., L.Y. Ke, L.L. Chang, C.C. Liu, Y.H. Hung, C.H. Lin, R.N. Li, W.C. Tsai, H.W. Liu, and J.H. Yen. 2010. Suppressor of cytokine signaling 1

gene expression and polymorphisms in systemic lupus erythematosus. *Lupus*. 19:696–702. http://dx.doi.org/10.1177/0961203309357437

Chaudhry, A., D. Rudra, P. Treuting, R.M. Samstein, Y. Liang, A. Kas, and A.Y. Rudensky. 2009. CD4$^+$ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science*. 326:986–991. http://dx.doi.org/10.1126/science.1172702

Durant, L., W.T. Watford, H.L. Ramos, A. Laurence, G. Vahedi, L. Wei, H. Takahashi, H.W. Sun, Y. Kanno, F. Powrie, and J.J. O'Shea. 2010. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity*. 32:605–615. http://dx.doi.org/10.1016/j.immuni.2010.05.003

Floess, S., J. Freyer, C. Siewert, U. Baron, S. Olek, J. Polansky, K. Schlawe, H.D. Chang, T. Bopp, E. Schmitt, et al. 2007. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*. 5:e38. http://dx.doi.org/10.1371/journal.pbio.0050038

Fontenot, J.D., J.P. Rasmussen, L.M. Williams, J.L. Dooley, A.G. Farr, and A.Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*. 22:329–341. http://dx.doi.org/10.1016/j.immuni.2005.01.016

Hanada, T., H. Yoshida, S. Kato, K. Tanaka, K. Masutani, J. Tsukada, Y. Nomura, H. Mimata, M. Kubo, and A. Yoshimura. 2003. Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. *Immunity*. 19:437–450. http://dx.doi.org/10.1016/S1074-7613(03)00240-1

Hori, S. 2010. c-Rel: a pioneer in directing regulatory T-cell lineage commitment? *Eur. J. Immunol*. 40:664–667. http://dx.doi.org/10.1002/eji.201040372

Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 299:1057–1061. http://dx.doi.org/10.1126/science.1079490

Inagaki-Ohara, K., T. Chinen, G. Matsuzaki, A. Sasaki, Y. Sakamoto, K. Hiromatsu, F. Nakamura-Uchiyama, Y. Nawa, and A. Yoshimura. 2004. Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation. *J. Immunol*. 173:1390–1398.

Isomäki, P., T. Alanärä, P. Isohanni, A. Lagerstedt, M. Korpela, T. Moilanen, T. Visakorpi, and O. Silvennoinen. 2007. The expression of SOCS is altered in rheumatoid arthritis. *Rheumatology (Oxford)*. 46:1538–1546. http://dx.doi.org/10.1093/rheumatology/kem198

Kim, H.P., and W.J. Leonard. 2007. CREB/ATF-dependent T cell receptor-induced *FoxP3* gene expression: a role for DNA methylation. *J. Exp. Med*. 204:1543–1551.

Kitoh, A., M. Ono, Y. Naoe, N. Ohkura, T. Yamaguchi, H. Yaguchi, I. Kitabayashi, T. Tsukada, T. Nomura, Y. Miyachi, et al. 2009. Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3$^+$ regulatory T cells. *Immunity*. 31:609–620. http://dx.doi.org/10.1016/j.immuni.2009.09.003

Koch, M.A., G. Tucker-Heard, N.R. Perdue, J.R. Killebrew, K.B. Urdahl, and D.J. Campbell. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat. Immunol*. 10:595–602. http://dx.doi.org/10.1038/ni.1731

Komatsu, N., M.E. Mariotti-Ferrandiz, Y. Wang, B. Malissen, H. Waldmann, and S. Hori. 2009. Heterogeneity of natural Foxp3$^+$ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc. Natl. Acad. Sci. USA*. 106:1903–1908. http://dx.doi.org/10.1073/pnas.0811556106

Lu, L.F., T.H. Thai, D.P. Calado, A. Chaudhry, M. Kubo, K. Tanaka, G.B. Loeb, H. Lee, A. Yoshimura, K. Rajewsky, and A.Y. Rudensky. 2009. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity*. 30:80–91. http://dx.doi.org/10.1016/j.immuni.2008.11.010

Lu, L.F., M.P. Boldin, A. Chaudhry, L.L. Lin, K.D. Taganov, T. Hanada, A. Yoshimura, D. Baltimore, and A.Y. Rudensky. 2010. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell*. 142:914–929. http://dx.doi.org/10.1016/j.cell.2010.08.012

Maloy, K.J., L. Salaun, R. Cahill, G. Dougan, N.J. Saunders, and F. Powrie. 2003. CD4$^+$CD25$^+$ T_R cells suppress innate immune pathology through cytokine-dependent mechanisms. *J. Exp. Med*. 197:111–119. http://dx.doi.org/10.1084/jem.20021345

Murai, M., P. Krause, H. Cheroutre, and M. Kronenberg. 2010. Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. *Mucosal Immunol*. 3:443–449. http://dx.doi.org/10.1038/mi.2010.27

Nakae, S., Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, I. Homma, K. Sekikawa, M. Asano, and Y. Iwakura. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity*. 17:375–387. http://dx.doi.org/10.1016/S1074-7613(02)00391-6

Oldenhove, G., N. Bouladoux, E.A. Wohlfert, J.A. Hall, D. Chou, L. Dos Santos, S. O'Brien, R. Blank, E. Lamb, S. Natarajan, et al. 2009. Decrease of Foxp3$^+$ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity*. 31:772–786. http://dx.doi.org/10.1016/j.immuni.2009.10.001

Onishi, Y., Z. Fehervari, T. Yamaguchi, and S. Sakaguchi. 2008. Foxp3$^+$ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc. Natl. Acad. Sci. USA*. 105:10113–10118. http://dx.doi.org/10.1073/pnas.0711106105

Rubtsov, Y.P., R.E. Niec, S. Josefowicz, L. Li, J. Darce, D. Mathis, C. Benoist, and A.Y. Rudensky. 2010. Stability of the regulatory T cell lineage in vivo. *Science*. 329:1667–1671. http://dx.doi.org/10.1126/science.1191996

Rudra, D., T. Egawa, M.M. Chong, P. Treuting, D.R. Littman, and A.Y. Rudensky. 2009. Runx-CBFbeta complexes control expression of the transcription factor Foxp3 in regulatory T cells. *Nat. Immunol*. 10:1170–1177. http://dx.doi.org/10.1038/ni.1795

Sakaguchi, S. 2004. Naturally arising CD4$^+$ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol*. 22:531–562. http://dx.doi.org/10.1146/annurev.immunol.21.120601.141122

Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell*. 133:775–787. http://dx.doi.org/10.1016/j.cell.2008.05.009

Scalapino, K.J., and D.I. Daikh. 2009. Suppression of glomerulonephritis in NZB/NZW lupus prone mice by adoptive transfer of ex vivo expanded regulatory T cells. *PLoS ONE*. 4:e6031. http://dx.doi.org/10.1371/journal.pone.0006031

Sharabi, A., Z.M. Sthoeger, K. Mahlab, S. Lapter, H. Zinger, and E. Mozes. 2009. A tolerogenic peptide that induces suppressor of cytokine signaling (SOCS)-1 restores the aberrant control of IFN-gamma signaling in lupus-affected (NZB x NZW)F1 mice. *Clin. Immunol*. 133:61–68. http://dx.doi.org/10.1016/j.clim.2009.06.010

Smith, E., M.A. Stark, A. Zarbock, T.L. Burcin, A.C. Bruce, D. Vaswani, P. Foley, and K. Ley. 2008. IL-17A inhibits the expansion of IL-17A-producing T cells in mice through "short-loop" inhibition via IL-17 receptor. *J. Immunol*. 181:1357–1364.

Sutton, C.E., S.J. Lalor, C.M. Sweeney, C.F. Brereton, E.C. Lavelle, and K.H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity*. 31:331–341. http://dx.doi.org/10.1016/j.immuni.2009.08.001

Suzue, K., T. Asai, T. Takeuchi, and S. Koyasu. 2003. In vivo role of IFN-gamma produced by antigen-presenting cells in early host defense against intracellular pathogens. *Eur. J. Immunol*. 33:2666–2675. http://dx.doi.org/10.1002/eji.200323292

Takimoto, T., Y. Wakabayashi, T. Sekiya, N. Inoue, R. Morita, K. Ichiyama, R. Takahashi, M. Asakawa, G. Muto, T. Mori, et al. 2010. Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. *J. Immunol*. 185:842–855. http://dx.doi.org/10.4049/jimmunol.0904100

Tanaka, K., K. Ichiyama, M. Hashimoto, H. Yoshida, T. Takimoto, G. Takaesu, T. Torisu, T. Hanada, H. Yasukawa, S. Fukuyama, et al. 2008. Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-gamma on STAT3 and Smads. *J. Immunol*. 180:3746–3756.

Tang, Q., J.Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C.A. Piccirillo, B.L. Salomon, and J.A. Bluestone. 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity*. 28:687–697. http://dx.doi.org/10.1016/j.immuni.2008.03.016

Tsuji, M., N. Komatsu, S. Kawamoto, K. Suzuki, O. Kanagawa, T. Honjo, S. Hori, and S. Fagarasan. 2009. Preferential generation of follicular B helper T cells from Foxp3$^+$ T cells in gut Peyer's patches. *Science*. 323:1488–1492. http://dx.doi.org/10.1126/science.1169152

Wan, Y.Y., and R.A. Flavell. 2007. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature*. 445:766–770. http://dx.doi.org/10.1038/nature05479

Wang, Y., A. Kissenpfennig, M. Mingueneau, S. Richelme, P. Perrin, S. Chevrier, C. Genton, B. Lucas, J.P. DiSanto, H. Acha-Orbea, et al. 2008. Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3$^+$ regulatory T cells. *J. Immunol.* 180:1565–1575.

Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3$^+$ regulatory T cell function. *Science*. 322:271–275. http://dx.doi.org/10.1126/science.1160062

Yang, X.O., R. Nurieva, G.J. Martinez, H.S. Kang, Y. Chung, B.P. Pappu, B. Shah, S.H. Chang, K.S. Schluns, S.S. Watowich, et al. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. 29:44–56. http://dx.doi.org/10.1016/j.immuni.2008.05.007

Yao, Z., Y. Kanno, M. Kerenyi, G. Stephens, L. Durant, W.T. Watford, A. Laurence, G.W. Robinson, E.M. Shevach, R. Moriggl, et al. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood*. 109:4368–4375. http://dx.doi.org/10.1182/blood-2006-11-055756

Yoshimura, A., T. Naka, and M. Kubo. 2007. SOCS proteins, cytokine signalling and immune regulation. *Nat. Rev. Immunol.* 7:454–465. http://dx.doi.org/10.1038/nri2093

Zhan, Y., G.M. Davey, K.L. Graham, H. Kiu, N.L. Dudek, T.W. Kay, and A.M. Lew. 2009. SOCS1 negatively regulates the production of Foxp3+ CD4+ T cells in the thymus. *Immunol. Cell Biol.* 87:473–480. http://dx.doi.org/10.1038/icb.2009.23

Zheng, Y., S. Josefowicz, A. Chaudhry, X.P. Peng, K. Forbush, and A.Y. Rudensky. 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 463:808–812. http://dx.doi.org/10.1038/nature08750

Zhou, X., S.L. Bailey-Bucktrout, L.T. Jeker, C. Penaranda, M. Martínez-Llordella, M. Ashby, M. Nakayama, W. Rosenthal, and J.A. Bluestone. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* 10:1000–1007. http://dx.doi.org/10.1038/ni.1774