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Suppressor of cytokine signaling (SOCS) proteins are key regulators of CD4+ T cell differentiation, and in particular, we have recently shown that SOCS2 inhibits the development of Th2 cells and allergic immune responses. Interestingly, transcriptome analyses have identified SOCS2 as being preferentially expressed in both natural regulatory T cells (Tregs) and inducible Tregs (iTregs); however, the role of SOCS2 in Foxp3+ Treg function or development has not been fully elucidated. In this study, we show that despite having no effect on natural Treg development or function, SOCS2 is highly expressed in iTregs and required for the stable expression of Foxp3 in iTregs in vitro and in vivo. Indeed, SOCS2-deficient CD4+ T cells upregulated Foxp3 following in vitro TGF-β stimulation, but failed to maintain stable expression of Foxp3. Moreover, in vivo generation of iTregs following OVA feeding was impaired in the absence of SOCS2 and could be rescued in the presence of IL-4 neutralizing Ab. Following IL-4 stimulation, SOCS2-deficient Foxp3+ iTregs secreted elevated IFN-γ and IL-13 levels and displayed enhanced STAT6 phosphorylation. Therefore, we propose that SOCS2 regulates iTreg stability by downregulating IL-4 signaling. Moreover, SOCS2 is essential to maintain the anti-inflammatory phenotype of iTregs by preventing the secretion of proinflammatory cytokines. Collectively, these results suggest that SOCS2 may prevent IL-4–induced Foxp3+ iTreg instability. Foxp3+ iTregs are key regulators of immune responses at mucosal surfaces; therefore, this dual role of SOCS2 in both Th2 and Foxp3+ iTregs reinforces SOCS2 as a potential therapeutic target for Th2-biased diseases. The Journal of Immunology, 2013, 190: 3235–3245.

**Regulation of Foxp3+ Inducible Regulatory T Cell Stability by SOCS2**

Camille A. Knosp,* Chris Schiering,† Shaun Spence,* Helen P. Carroll,* Hendrick J. Nel,‡ Megan Osbourn,* Ruaidhri Jackson,* Oksana Lyubomska,* Bernard Malissen,‡ Rebecca Ingram,* Denise C. Fitzgerald,* Fiona Powrie,† Padraic G. Fallon,‡ James A. Johnston,*1 and Adrien Kissenpfennig*

Suppressor of cytokine signaling (SOCS) proteins are key regulators of CD4+ T cell differentiation, and in particular, we have recently shown that SOCS2 inhibits the development of Th2 cells and allergic immune responses. Interestingly, transcriptome analyses have identified SOCS2 as being preferentially expressed in both natural regulatory T cells (Tregs) and inducible Tregs (iTregs); however, the role of SOCS2 in Foxp3+ Treg function or development has not been fully elucidated. In this study, we show that despite having no effect on natural Treg development or function, SOCS2 is highly expressed in iTregs and required for the stable expression of Foxp3 in iTregs in vitro and in vivo. Indeed, SOCS2-deficient CD4+ T cells upregulated Foxp3 following in vitro TGF-β stimulation, but failed to maintain stable expression of Foxp3. Moreover, in vivo generation of iTregs following OVA feeding was impaired in the absence of SOCS2 and could be rescued in the presence of IL-4 neutralizing Ab. Following IL-4 stimulation, SOCS2-deficient Foxp3+ iTregs secreted elevated IFN-γ and IL-13 levels and displayed enhanced STAT6 phosphorylation. Therefore, we propose that SOCS2 regulates iTreg stability by downregulating IL-4 signaling. Moreover, SOCS2 is essential to maintain the anti-inflammatory phenotype of iTregs by preventing the secretion of proinflammatory cytokines. Collectively, these results suggest that SOCS2 may prevent IL-4–induced Foxp3+ iTreg instability. Foxp3+ iTregs are key regulators of immune responses at mucosal surfaces; therefore, this dual role of SOCS2 in both Th2 and Foxp3+ iTregs reinforces SOCS2 as a potential therapeutic target for Th2-biased diseases. The Journal of Immunology, 2013, 190: 3235–3245.
SOCS2-deficient iTregs secreted elevated IFN-γ and IL-13 levels and had increased STAT6 phosphorylation following IL-4 stimulation. Moreover, blocking of IL-4 both in vitro and in vivo restored iTreg stability in SOCS2-deficient T cells. Therefore, our data suggest that SOCS2 is an essential regulator of Foxp3+ iTreg stability and plasticity.

Materials and Methods

Mice

All experiments used 8–16-wk-old, sex- and age-matched mice that were housed under specific pathogen-free conditions. Socs2−/− mice were a generous gift from D.J. Hilton (Royal Melbourne Hospital, VIC, Australia) and were generated on a C57BL/6 background as previously described (26). C57BL/6J Foxp3eGFP (B6-Foxp3eGFP) mice have been previously described (27). C57BL/6 mice were purchased from Harlan Laboratories, and CD45.1 and OT-II strain mice were purchased from The Jackson Laboratory. Foxp3eGFP and OT-II mice were subsequently crossed to Socs2−/− mice in-house to generate Socs2−/− × Foxp3eGFP and Socs2−/− × OT-II mice lines, respectively. All animal experiments were performed in compliance with UK Home Office regulations and approved by the Queen’s University Ethical Review Committee.

Preparation of lymphocyte cell suspensions

Spleen, lymph nodes (LN), and thymus were aseptically removed from mice, and single-cell suspensions were prepared, as previously described (16). Intact small intestine was extracted, cleaned, and dissected. Tissue pieces were disrupted in 5 ml trypsin-EDTA (0.005%; Life Technologies) diluted in PBS-2% FCS and mechanically agitated for 15 min at 37°C. The resulting cell suspension was then filtered through 100-μm nylon cell strainers (BD Falcon), centrifuged 10 min at 700 × g, and washed in 10 ml RPMI-1640, 2% FCS. The cells were then subjected to a Percoll gradient (Sigma-Aldrich) of 75% (3 ml)/40% (4 ml), whereas the cells were resuspended in 3 ml 30%. Following 40 min centrifugation at 200 × g, cells present at the 75% and 40% Percoll interface were removed and washed in 30 ml PBS-2% FCS.

Flow cytometry

Surface marker expression was assessed by flow cytometry using CD3, CD4, CD8, CD25, CD45R1, CD45.1, and CD45.2 Abs (BD Biosciences and eBioscience). For Foxp3 staining, cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) as recommended by the manufacturer. For phospho-STAT6 analysis, the cells were fixed after stimulation, permeabilized with the Perm III buffer (BD Biosciences), and stained with Abs specific for phospho-STAT6 (BD Biosciences) according to the Phosflow instructions (BD Biosciences). Data were collected on a BD FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star). All flow cytometry analyses were performed following live cell gating and cell doublet exclusion from FSC/SSC profiles.

Cell purification and sorting

CD4+CD25+ and CD4+CD25− T cells were magnetically isolated by negative selection for CD4 and subsequent positive selection for CD25 (Miltenyi Biotec). CD4+Foxp3+, CD4Foxp3− T cells, and naive CD4+CD25−CD45RB+ were sorted from Foxp3eGFP and Socs2−/− × Foxp3eGFP to >99% purity with a BD FACS Aria II (BD Biosciences) using anti-CD4, anti-CD45R1, and anti-CD25 Abs (eBioscience). iTregs were obtained from presorted CD4+Foxp3+ lymphocytes, and stimulated for 3 d with plate-bound anti-CD3 (2 μg/ml; clone 145-2C11; eBioscience), anti-CD28 (1 μg/ml; clone 37.51; eBioscience), and TGF-β (3 ng/ml; R&D Systems). At day 3, cells were stained with CD4 Ab, and the iTreg population was isolated based on CD4 and Foxp3-eGFP expression by FACS.

Proliferation assay

Cell proliferation was evaluated either by CFSE or CellTrace Violet (CTV) dilution (Invitrogen). Briefly, cells were resuspended in serum-free RPMI-1640 and an equal volume of serum-free RPMI-1640 containing either 10 μM CFSE or 5 μM CTV was added. Cells were then vigorously mixed and incubated at 37°C for either 10 min (CFSE) or 20 min (CTV). Cells were then washed and resuspended in appropriate medium.

Cell culture

Unless otherwise specified, cells were resuspended at 1 × 106 cells/ml in complete RPMI-1640 medium supplemented with 10% FCS, penicillin, and streptomycin (1%; PAA Laboratories), t-glutamine (200 mM; PAA Laboratories), and 2-ME (50 μM; Sigma-Aldrich). Cells were then stimulated with plate-coated anti-CD3 (2 μg/ml; eBioscience) and anti-CD28 (1 μg/ml; eBioscience). CD4+CD25+ Treg proliferation was triggered by adding exogenous recombinant human (rh)IL-2 (250 U/ml; R&D Systems), Foxp3 expression was induced in vitro by stimulating CD4+CD25+ T cells with rhTGF-β (1–3 ng/ml; R&D Systems) and, in some assays, supplemented with neutralizing IL-4 Ab (20 μg/ml; R&D Systems). iTregs were restimulated with rhIL-2 (5 U/ml; R&D Systems), recombinant murine IL-12 (50 ng/ml; R&D Systems), rhIL-6 (50 U/ml; R&D Systems), or recombinant murine IL-4 (5 μg/ml; R&D Systems).

Suppressive assay

CD4+Foxp3+ conventional T cells (Tconv) were purified from Foxp3eGFP or Socs2−/− × Foxp3eGFP and labeled with CTV, as described above. Foxp3+ Tregs or Foxp3+ iTregs were sorted as described above and cocultured at the indicated ratio with 50,000 Tconv cells per condition. A total of 100,000 APCs obtained by irradiating (120 Gy) splenocytes depleted of CD4+ and CD8+ T cells were also added to the assay. Cells were then stimulated with soluble CD3 (2 μg/ml; eBioscience) and CD28 (1 μg/ml; eBioscience). After 3 d stimulation, cells were harvested, washed, and stained with CD4 Ab, Tconv proliferation was evaluated by flow cytometry, and the percentage of suppression was calculated.

In vivo induction of Foxp3

In vivo iTreg development was induced by oral administration of OVA. Briefly, spleens and LN were harvested from OT-II and Socs2−/− × OT-II mice. CD4+ T cells were enriched by negative selection using the EasySep mouse CD4+ T cell enrichment kit (StemCell Technologies). Enriched CD4+ T cells were subsequently stained with anti-CD25 and anti-CD45RB Abs (eBioscience) and sorted to >99% purity with a FACS Aria II (BD Biosciences). Cells were then washed in PBS, and up to 4 × 106 cells were retro-orbitally injected into congenic CD45.1 mice. The following day, drinking water was supplemented with 3% OVA (Sigma-Aldrich) and 5% sucrose (Sigma-Aldrich), or 5% sucrose only for the control group, and water was changed on a daily basis. After 5 d, mice were sacrificed, and small intestine, mesenteric LN (mLN), and spleen were removed. Lymphocytes were isolated as previously described, stained for CD4, CD45.1, CD45.2, and Foxp3 expression, and analyzed by flow cytometry. For in vivo IL-4 depletion experiments, IL-4 was neutralized through injection of mice with 250 μg anti-IL-4 mAb (clone 1B11; American Type Culture Collection) or IgG3 isotype control i.v. in CD45.1 congenic 24 h prior to adoptive transfer of OT-II or Socs2−/− × OT-II naive T cells and every subsequent 24 h for 5 d since OVA feeding.

Transfer of naive CD4+ T cell into Il23a−/− Rag1−/− mice leads to spontaneous development of iTreg in vivo (28). As a second model of iTreg induction, CD4+CD25+ Foxp3+CD45RB− naive T cells were sorted from Foxp3eGFP or Socs2−/− × Foxp3eGFP mice, and 4 × 105 cells were injected i.p. into Il23a−/− Rag1−/− mice, as previously described (29). Mice were sacrificed 8 wk posttransfer, and lymphocytes were extracted from the colon and analyzed by flow cytometry.

Immunoblotting and ELISA

Cells were lysed in a buffer composed of Brij 97 (0.875% v/v), Tris (50 mM), NaCl (150 mM), EDTA (5 mM), Na3V04 (1 mM), leupeptin (10 μg/ml), aprotinin (10 μg/ml), and PMSF (1 mM) for 15 min on ice. Cell lysates were then clarified by centrifugation and analyzed by immunoblotting as previously described (30). SOCS2 expression was analyzed using an anti- SOCS2 Ab (Cell Signaling Technology), and protein loading was verified by blotting for γ-tubulin (Sigma-Aldrich). IL-4, IL-5, IL-13, and IFN-γ secretion in cell-culture supernatants was analyzed by ELISA (R&D Systems).

Statistical analysis

Data were analyzed with Prism4 (GraphPad). Statistical differences were determined by two-tailed unpaired t test as appropriate.

Results

SOCS2 deficiency does not affect steady-state Foxp3+ Treg numbers

Socs2−/− mice present with normal CD4+ T cell development (16, 31). Interestingly, several groups have since characterized the Foxp3+ Treg transcriptome and revealed that SOCS2 is prefer-
entially expressed in Foxp3+ Treg cells compared with other T cell subsets, both in the thymus and in the periphery (23–25). We therefore investigated whether SOCS2 deficiency would affect Foxp3+ nTreg development in vivo. However, following examination of the thymus, spleen, mLN, and other peripheral LN (axillary, brachial, and inguinal), similar frequencies (Fig. 1A) and absolute numbers (Fig. 1B) of CD4+ Foxp3+ T cells in Foxp3-eGFP and Socs2−/− × Foxp3-eGFP mice were found, suggesting that SOCS2 does not regulate Foxp3+ Treg development under steady-state conditions.

**SOCS2 deficiency does not affect nTreg proliferation and suppressive function**

Sugimoto and colleagues (23) previously showed that CD4+CD25− T cells retrovirally transduced with SOCS2 became anergic and exerted some suppressive function, suggesting that these cells acquired a Treg-like phenotype. We thus examined whether SOCS2 deficiency would affect Treg cell division and their ability to inhibit CD4+CD25− T cell proliferation. Peripheral Tregs were purified by magnetic selection from wild-type (WT) C57BL/6J and Socs2−/− mice, and their ability to proliferate following in vitro TCR stimulation in the presence of exogenous IL-2 was evaluated by CFSE dilution. WT and Socs2−/− Foxp3+CD4+ T cells strongly proliferated, and the frequency of dividing SOCS2-deficient CD4+Foxp3+ Tregs was comparable to control WT cells (Fig. 2A). Subsequently, we assessed the suppressive ability of SOCS2-deficient Tregs in vitro by evaluating the proliferation of CTV-labeled CD4+Foxp3− T cells cocultured with various ratios of WT or Socs2−/− CD4+ Foxp3+ T cells. Addition of either WT or Socs2−/− Tregs inhibited CD4+Foxp3− proliferation in a dose-dependent manner (Fig. 2B), with Socs2−/− Foxp3+ Tregs exerting comparable suppression to WT Tregs at all ratios examined (Fig. 2C). Importantly, to exclude the possibility that the lack of SOCS2 rendered CD4+Foxp3− T cells more resistant or more susceptible to Treg suppression, we also examined the ability of Socs2−/− Tregs to inhibit WT CD4+Foxp3+ T cell proliferation and of WT Tregs to suppress Socs2−/− CD4+Foxp3+ T cells. In both cases, the suppression exerted was comparable to inhibition of WT CD4+Foxp3− T cell by WT Tregs for all ratios examined (Fig. 2C), suggesting that SOCS2 does not regulate Treg suppressive function. Moreover, examination of CD25 and CTLA-4 expression on Foxp3+ Tregs did not reveal any difference between WT and Socs2−/− cells (data not shown). Therefore, SOCS2-deficient Foxp3+ Tregs presented with normal proliferation, suppressive function, and expression of Treg markers, highlighting that SOCS2 has a limited role in regulating Foxp3+ Treg function and phenotype.

**FIGURE 1.** SOCS2 does not affect Foxp3+ numbers under steady state. Lymphocytes were isolated from thymus, spleen, pooled peripheral axillary, brachial, and mLN from Socs2−/− × Foxp3-eGFP and Foxp3-eGFP mice, stained with anti-CD4, and analyzed by flow cytometry. (A) Representative dot plots of cells gated on CD4+ lymphocytes. Indicated numbers represent the percent of CD4+Foxp3+ cells within the CD4+ population. (B) Graphs representing absolute numbers of CD4+ Foxp3+ cells in two groups of age- and sex-matched mice (WT n = 7–10; Socs2−/− n = 6–8). The differences between the two groups were compared using the Student t test.
FIGURE 2. SOCS2 deficiency does not affect peripheral Foxp3+ Treg phenotype. (A) CD4+CD25+ lymphocytes purified with magnetic beads from WT and Socs2−/− mice were labeled with CFSE and stimulated with plate-bound anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) in the presence of IL-2 (250 U/ml). After 72 h, cells were stained with anti-CD4 and anti-Foxp3, and CD4+Foxp3+ T cell proliferation was evaluated by CFSE dilution. Histograms gated on CD4+ Foxp3+ T cells are representative of triplicates, and the experiment was performed twice with similar results. (B and C) CD4+Foxp3+ (Treg) and CD4+Foxp3− (Tconv) cells were sorted from Socs2−/− × Foxp3GFP and Foxp3GFP mice. Tconv were then labeled with CTV and cocultured with Tregs at different Treg/Tconv ratios. At day 3, cells were stained with anti-CD4 and analyzed by flow cytometry. (B) Representative histograms of Tconv proliferation. (C) Graph showing the mean suppression from triplicates, at different ratios of WT Tregs on WT or Socs2−/− Tconv and of Socs2−/− Tregs on WT or Socs2−/− Tconv. Experiments were performed twice with similar results.

**SOCS is highly expressed in Foxp3+ iTreg cells**

SOCS2 deficiency did not affect peripheral Foxp3+ nTreg numbers or phenotype, and this was surprising given the high levels of SOCS2 mRNA detected in these cells in previous studies (23, 24). Interestingly, Haribhai and colleagues (25) found that SOCS2 mRNA levels were increased in Foxp3+ iTregs generated in vitro compared with ex vivo peripheral Foxp3+ Tregs. Therefore we examined SOCS2 protein levels in various CD4+ subsets. As shown in Fig. 3, we could not detect SOCS2 protein expression in freshly isolated CD4+ T cells or Foxp3+ Tregs. However, SOCS2 was highly expressed in in vitro–derived WT iTregs, suggesting that SOCS2 may be important for iTreg development and/or function. Importantly, naive CD4+ T cells stimulated for 3 d with anti-CD3/CD28 Abs did not express SOCS2, implying that TGF-β signaling may directly promote SOCS2 expression in iTregs rather than TCR stimulation alone.

**SOCS2-deficient iTregs failed to maintain Foxp3 expression in vitro**

Because SOCS2 is highly expressed in Foxp3+ iTregs, we hypothesized that SOCS2 might be important for iTreg function, and we therefore first examined whether SOCS2 deficiency would affect Foxp3 induction in CD4+ T cells. Purified WT and Socs2−/− CD4+CD25+ T cells were stimulated with different concentrations of TGF-β and Foxp3 expression levels examined after 3 and 5 d in culture. Following 3 d of culture, the frequency of Foxp3+ iTreg was slightly lower in the absence of SOCS2 when compared with WT (Fig. 4A), although this was not statistically significant (Fig. 4B). However, by day 5, Socs2−/− iTreg numbers were dramatically reduced (Fig. 4A, 4B; p < 0.01-0.001) compared with WT CD4+ T cells that had maintained Foxp3 expression. Therefore, Socs2−/− CD4+ T cells can efficiently induce Foxp3, but they are unable to maintain Foxp3 expression. Furthermore, although increased concentrations of TGF-β could partially rescue the loss of Foxp3 expression, iTreg numbers were still significantly lower in the absence of SOCS2 compared with WT controls (Fig. 4B).

We consequently examined the cytokine secretion profile of WT and Socs2−/− CD4+CD25+ T cells stimulated with TGF-β following 3 and 5 d of culture. Significantly higher levels of IL-4, IL-13, and IFN-γ were detected in the absence of SOCS2 (Fig. 4C). This correlated with enhanced proliferation of Socs2−/− CD4+Foxp3+ T cells as shown by CFSE dilution (Supplemental Fig. 1) and suggested that Socs2−/− iTregs might have impaired suppressive function. Indeed, failure to inhibit CD4+Foxp3+ T cell proliferation would result in a relative decrease of Foxp3+ iTreg frequency, whereas increased secretion of inflammatory cytokines would prevent further induction of Foxp3. However, examination of Socs2−/− iTreg suppressive function in vitro did not reveal any difference with WT iTregs at all ratios examined (Supplemental Fig. 2), suggesting that although Socs2−/− iTregs could be induced in vitro, they were unable to maintain Foxp3 expression, and this was not due to an inherent defective suppressive function of Socs2−/− Foxp3+ iTregs. Therefore, we believe that in the absence of SOCS2, the increased proliferation of CD4+Foxp3+ T cells and subsequent secretion of proinflammatory cytokines is the direct consequence of the reduction in number of iTreg cells.

**Reduced iTreg numbers in the absence of SOCS2 in vivo**

To test the impact of SOCS2 deficiency on Foxp3 induction in vivo, we used a well-established model of oral tolerance induced by Ag (OVA) feeding (32–34). Naïve CD4+ CD45.2+ T cells purified from Socs2−/− × OT-I and OT-II control mice were transferred into CD45.1 congenic mice that were subsequently fed with OVA to induce Foxp3 expression. Following 5 d of OVA oral administration, mice were sacrificed, and the frequency of CD4+Foxp3+ cells in the small intestine, mLN, and spleen was significantly reduced (Fig. 4). This correlated with enhanced proliferation of CD4+Foxp3+ T cells as shown by CFSE dilution (Supplemental Fig. 1) and suggested that Socs2−/− iTregs might have impaired suppressive function. Indeed, failure to inhibit CD4+Foxp3+ T cell proliferation would result in a relative decrease of Foxp3+ iTreg frequency, whereas increased secretion of inflammatory cytokines would prevent further induction of Foxp3. However, examination of Socs2−/− iTreg suppressive function in vitro did not reveal any difference with WT iTregs at all ratios examined (Supplemental Fig. 2), suggesting that although Socs2−/− iTregs could be induced in vitro, they were unable to maintain Foxp3 expression, and this was not due to an inherent defective suppressive function of Socs2−/− Foxp3+ iTregs. Therefore, we believe that in the absence of SOCS2, the increased proliferation of CD4+Foxp3+ T cells and subsequent secretion of proinflammatory cytokines is the direct consequence of the reduction in number of iTreg cells.
evaluated. The transferred cells were identified based on CD45.2 expression (CD4+CD45.2+CD45.12), and the percentage of induction in transferred cells was established on the basis of Foxp3 expression in this population. The highest frequency of CD45.2+ cells was found in the small intestine at the primary site of stimulation (data not shown), consistent with previous studies (33, 34). iTreg frequencies were also found to be higher in the small intestine and mLN when compared with the spleen (Fig. 5A). Strikingly, Foxp3 induction was markedly lower in the absence of SOCS2, with up to 60% reduction in the small intestine and mLN when compared with animals with transferred WT OT-II controls (Fig. 5A). Moreover, the difference between the two groups was statistically significant in the three organs examined (Fig. 5C).

To further test whether SOCS2 would affect iTreg development in a different experimental setting of in vivo expansion of iTreg cells, we examined their effect in a colitis model. In this model, naive CD4+CD25−CD45RBhi sorted from WT and Soxs22/2 mice were transferred into Il23a2/2Rag12/2 mice that lack IL-23 (35). Indeed, Il23a2/2Rag12/2 mice present with an increased accumulation/differentiation of iTreg cells due to the absence of IL-23, which leads to protection from colitis symptoms (28). Transfer of Soxs22/2 but not WT CD4+ T cells induced weight loss in Il23a2/2Rag12/2 mice, suggesting that the reduced number of Soxs22/2 iTreg failed to protect the mice from colitis-like disease (Supplemental Fig. 3A). Moreover, as shown in Supplemental Fig. 3B, up to 2% of naive WT CD4+ T cells upregulated Foxp3 following transfer into Il23a2/2Rag12/2 mice, whereas significantly less SOCS2-deficient CD4+ T cells expressed Foxp3. Therefore, the development of iTreg cells was impaired in the absence of SOCS2 in two independent established in vivo models of iTreg induction. These data demonstrate that SOCS2 is an important regulator of iTreg development both in vitro and in vivo.

**Figure 3.** SOCS2 is highly expressed in iTreg. Freshly isolated Tregs (CD4+Foxp3+) and Tconvs (CD4+Foxp3−) were sorted from Soxs22/2FloxP3eGFP and Foxp3eGFP mice and directly lysed. iTregs were generated and sorted as described in the Materials and Methods, whereas CD4+Foxp3+ T cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) for 3 d (activated Tconv). After lysis, SOCS2 expression in each sample was examined by immunoblotting (IB) with anti-SOCS2 and anti-γ-tubulin. This experiment was done three times with similar results.

**Figure 4.** SOCS2-deficient Foxp3+ iTregs fail to maintain Foxp3 expression in vitro. CD4+CD25− cells were purified with magnetic beads from lymphocytes extracted from Soxs22/2 mice and WT mice. The cells were then labeled with CFSE and stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) in the presence of IL-2 (5 U/ml) and different concentrations of TGF-β. (A) Representative dot plots of CD4+ cells stimulated with 1 ng/ml of TGF-β and stained with anti-CD4 and anti-Foxp3 after 3 or 5 d. (B) Mean from triplicates of the frequency of Foxp3+ cell within CD4+ population of CD4+CD25− cells stimulated for 3 or 5 d with the indicated concentrations of TGF-β. (C) Secretion of IL-4, IL-13, and IFN-γ by CD4+CD25− cells stimulated with 1 ng/ml of TGF-β, evaluated by ELISA. The differences between groups were compared using the Student t test. **p < 0.01, ***p < 0.001.
IL-4 mediates Socs2−/− iTreg instability

SOCS2 clearly affects iTreg stability, and, based on our in vitro results (Fig. 4A, 4B), we hypothesized that Socs2−/− iTregs may not be as stable due to a possible impairment in the regulation of cytokine signaling or responses. Although Foxp3 expression stability in vivo is controversial (36, 37), there is some evidence that under specific conditions, Foxp3+ Tregs can actively secrete proinflammatory cytokines, such as IFN-γ or IL-17 (38), and also lose Foxp3 expression (39). To address our hypothesis, we sorted in vitro–derived WT and Socs2−/− Foxp3+ iTregs from Foxp3GFP

FIGURE 5. SOCS2 deficiency affects iTreg numbers in vivo. Naïve CD45.2+CD4+CD25− CD45RBhi T cells were sorted from Socs2−/− OT-II or OT-II mice, and 4 × 10^6 were retro-orbitally injected into congenic CD45.1 mice. After 5 d of OVA oral administration, mice were sacrificed, and lymphocytes extracted from the spleen, mLN, and small intestines were stained for CD4, CD45.1, CD45.2, and Foxp3 expression. Representative density plots representives of Foxp3 expression gated on CD4+CD45.2+CD45.1− population from mice fed with OVA and sucrose (A) or sucrose only (B). (C) Graphs representing iTreg frequencies in two groups of CD45.1 mice fed with OVA that received OT-II or Socs2−/− OT-II cells (n = 4–7). The differences between the two groups were compared using the Student t test. *p < 0.05, **p < 0.01, ***p < 0.001.
mice and examined their stability upon stimulation with various cytokines. As expected, IL-12, IL-6, and IL-4 promoted the loss of Foxp3 expression in iTregs from WT mice when compared with IL-2, with IL-4 being the most potent (Fig. 6A, Supplemental Fig. 4). Interestingly, SOCS2 deficiency did not affect iTreg stability when stimulated with IL-2, IL-6, or IL-12 (Fig. 6A, Supplemental Fig. 4), but following IL-4 stimulation, the frequency of Socs2−/− iTregs was significantly reduced compared with WT iTregs (Fig. 6A, 6B). Moreover, this was associated with markedly enhanced IL-13 secretion (Fig. 6C), suggesting that IL-4 repolarized Socs2−/− iTregs toward a Th2 phenotype. Interestingly, Socs2−/− iTregs secreted constitutively higher IFN-γ levels compared with WT iTregs, regardless of the stimulating cytokines used (Fig. 6D), suggesting that SOCS2 constitutively repressed IFN-γ secretion.

To confirm an in vitro role of IL-4 in mediating Socs2−/− iTreg instability, we repeated TGF-β–mediated Foxp3 induction in CD4+Foxp3− T cells in the presence of a neutralizing IL-4 Ab. As shown in Fig. 6E, blocking of IL-4 prevented the reduction of iTreg frequency observed in the absence of SOCS2, further demonstrating that IL-4 plays a key role in this process. This phenomenon was also confirmed in vivo in a model of OVA oral tolerance (Fig. 6F). Naïve CD4+ CD45.2+ T cells purified from Socs2−/− × OT-II and OT-II control mice were transferred into CD45.1 congenic mice that were subsequently fed OVA over 5 d. CD45.1 animals receiving Socs2−/− × OT-II and OT-II CD44 naïve T cells were then treated with either IL-4–neutralizing Ab or an IgG1 isotype control. After 5 d, small intestine, mLN, and spleen lymphocytes were isolated from all four groups and the frequency of iTregs assessed. Importantly, blocking of IL-4 in vivo rescued iTreg stability in mice that had received Socs2−/− × OT-II T cells compared with the same cohort of animals treated with the isotype control, in which, again, iTreg instability was observed. Socs2−/− × OT-II iTreg frequencies and absolute numbers were found to be significantly increased following blocking of IL-4 in the small intestine, mLN, and spleen (Fig 6F). Blocking of IL-4 also increased numbers of both OT-II and Socs2−/− × OT-II iTregs compared with isotype treated animals, which was consistent with a previous publication (32).

Collectively, our data demonstrate that Socs2−/− iTregs failed to maintain Foxp3 expression when stimulated with IL-4 and constitutively secreted IFN-γ. Of note, the fact that Socs2−/− CD4+ T cells secreted significantly higher IL-4 compared with WT CD4+ T cells may accelerate this process, both in vitro and in vivo systems (16).

**SOCS2 regulates IL-4–induced STAT6 activation in Foxp3+ iTreg**

SOCS2 proteins are key regulators of STAT activation, and many studies have now shown that STATs play a key role in CD4+ T cell polarization (40). In particular, STAT6 was found to bind to the Foxp3 promoter and to inhibit TGF-β–mediated Foxp3 induction (9, 10, 41). Because Socs2−/− iTregs were more susceptible to IL-4–mediated Foxp3 instability, we hypothesized that SOCS2 might regulate IL-4 signaling in Foxp3+ iTregs. We thus examined STAT6 activation following IL-4 stimulation in WT and Socs2−/− CD4+ T cells prestimulated for 48 h with TGF-β. STAT6 phosphorylation levels were significantly increased in the absence of SOCS2 compared with WT cells, both after 30 min and 2 h stimulation with IL-4 (Fig. 7A, 7B). Therefore, IL-4–mediated STAT6 signaling is enhanced in Socs2−/− CD4+ T cells prestimulated with TGF-β, demonstrating that SOCS2 prevents IL-4–induced Foxp3+ iTreg plasticity. Altogether, these results show that high expression of SOCS2 contributes to iTreg stability by inhibiting STAT6 activation following IL-4 stimulation.

**Discussion**

Several studies have reported that Foxp3+ Tregs isolated from the thymus and also from secondary lymphoid organs expressed high levels of SOCS2 mRNA (23–25), and consequently, we hypothesized that SOCS2 may play an important role in regulating Foxp3+ Treg development or function. However, we found that Socs2−/− mice displayed normal Foxp3+ Treg numbers in the thymus and periphery under steady-state conditions and that SOCS2-deficient Foxp3+ Tregs had a normal phenotype. Moreover, ex vivo Foxp3+ Tregs purified from Socs2−/− mice exhibited normal proliferation and exerted similar suppressive function when compared with WT Foxp3+ Tregs. This was consistent with the lack of SOCS2 protein expression in purified WT Foxp3+ Tregs and suggested that SOCS2 has a nonessential role in these cells. However, we found that SOCS2 is highly expressed in iTregs and that the absence of SOCS2 selectively affected their stability both in vitro and in vivo. Indeed, when stimulated with TGF-β, naïve Socs2−/− CD4+ T cells upregulated Foxp3 but failed to maintain its expression in vitro. Socs2−/− iTregs were less stable than WT iTregs when stimulated with IL-4 most likely due to hyperactivation of STAT6 and secreted higher levels of IL-13 and, surprisingly, also IFN-γ. Increased IFN-γ production was unexpected, as it was not mediated by a specific cytokine. One possible explanation might come from our previous finding that STAT5 activation was enhanced in the absence of SOCS2 (16). Indeed, it was recently proposed that IL-2–mediated STAT5 activation is required for optimal secretion of IFN-γ (42). Therefore, Socs2−/− iTregs could also be hyperresponsive to IL-2, and this may mediate the increased IFN-γ secretion we observed (43, 44). Nevertheless, the enhanced IFN-γ secretion by the Socs2−/− iTreg did not affect their stability, and therefore, we propose that IL-4 is the main cause of Socs2−/− iTreg instability. Indeed, addition of an Ab neutralizing IL-4 was sufficient to restore iTreg numbers both in vivo and in vitro. However, our present results cannot exclude that cytokines other than IL-2, IL-6, and IL-12 may also contribute to iTreg instability in the absence of SOCS2. It is possible that the elevated secretion of IL-13 and IFN-γ by Socs2−/− iTreg would ultimately also affect their stability, but our present results do not allow us to draw conclusions on this point. However, this study clearly shows that SOCS2 is required to maintain the anti-inflammatory phenotype of iTregs.

SOCS2 deficiency inhibits iTreg development in vivo, as naïve CD4+ T cells isolated from Socs2−/− × OT-II mice displayed impaired ability to upregulate Foxp3 and maintain a stable Treg population following OVA administration in a model of oral tolerance. Surprisingly, Socs2−/− mice do not develop any spontaneous autoimmune disorders (26), likely due to the fact that the pool of peripheral Foxp3+ Tregs, that includes natural Tregs, is not affected by SOCS2 deficiency. Moreover, Socs2−/− mice are ubiquitous knockouts (26), and the lack of SOCS2 in other immune cells may differentially affect disease development. The generation of mice lacking SOCS2 solely in Foxp3+ Treg cells would therefore be important to study whether the impaired stability of iTregs would confer an increased susceptibility to autoimmune diseases. Importantly, although Socs2−/− mice present with no obvious immune deficiencies under steady-state conditions (26), SOCS2 clearly plays a key regulatory role when the immune system is challenged. Indeed, Socs2−/− mice succumb to Toxoplasma gondii infection (45) and are more susceptible to type 2 allergic responses (16). Although other immune cells mediate...
these effects, the impaired ability to generate a stable population of iTregs cells may also contribute to the excessive inflammation observed in these studies.

We previously showed that SOCS2 inhibits Th2 differentiation, and interestingly, there are accumulating studies showing the close relationship between Foxp3+ Tregs and Th2 cells (46).
is illustrated by the ability of Foxp3 and GATA-3 to bind to each other (9). It was recently demonstrated that GATA-3 expression is essential for Foxp3+ Treg function (47, 48), as GATA-3 inhibits T-bet and retinoic acid–related orphan receptor γt expression (48), thus directly contributing to the stability of Foxp3+ Treg phenotype. Furthermore, GATA-3–deficient Foxp3+ Treg fail to maintain high Foxp3 levels and accumulate at inflammatory sites, leading to spontaneous inflammatory disorders in mice (47, 48). Importantly, Foxp3 directly regulates GATA-3 transcriptional activity and in particular prevents IL-5 induction (41, 49). It is also likely that GATA-3 expression levels are tightly regulated in Foxp3+ Tregs, and this might be directly controlled by STAT6, in fact STAT6 is able to bind the Foxp3 promoter, and to prevent Foxp3 induction following TGF-β stimulation (10). Moreover, our results show that Foxp3+ iTregs can lose Foxp3 expression following IL-4 stimulation in vitro, suggesting that STAT6 can repress Foxp3 expression. Interestingly, in the absence of SOCS2, failure to inhibit STAT6 activation is associated with the loss of Foxp3 expression and acquisition of a Th2 phenotype, likely due to increased GATA-3 levels. Therefore, a tight regulation of STAT6 activation and GATA-3 expression levels seems to be required for a stable iTreg phenotype, and SOCS2 may play a key role in this process.

The fate of CD4+ T cells is governed by the cytokines they encounter, leading to the increased expression of master regulators, determining in particular their cytokine secretion profile (53). As reported by many studies, CD4+ lineages are not firmly committed as initially thought, but have some degree of plasticity, allowing coexpression of master transcriptional regulators and cytokines specific for other lineages (54).Interestingly, SOCS proteins seem to adjust the balance between the different CD4+ lineages (14). Indeed, SOCS1 and SOCS3 promote Th17 and Th2 differentiation, respectively, by inhibiting Th1 differentiation (15, 18), whereas SOCS2 favors the development of Th17 cells at the expense of Th2 differentiation (16). It is therefore plausible that SOCS proteins play a significant role in the regulation of CD4+ T cell plasticity. Although controversial (36, 37), there is accumulating evidence that Foxp3+ Tregs do not always stably express Foxp3 and can repolarize toward other lineages (38, 39, 55). Importantly, several studies have now demonstrated that SOCS1 ensures Foxp3+ Treg stability by limiting STAT1 activation (21, 22). Indeed Socs1−/− Foxp3+ Tregs constitutively failed to maintain Foxp3 expression in vivo due to a hyperactivation of IFN-γ–mediated STAT1 (22). Moreover, SOCS1−/− Tregs secreted IFN-γ and IL-17, which limited their suppressive function (21, 22). Similarly, we found that SOCS2 prevented the loss of Foxp3 expression in iTregs by regulating IL-4 signaling and preventing IFN-γ and IL-13 secretion. This suggests that SOCS proteins maintain Foxp3+ Treg stability but also prevent their repolarization toward other lineages. Therefore, our study extends our understanding of how SOCS proteins regulate the stability of Foxp3+ Treg cells and plasticity of CD4+ T cells. In conclusion, we propose that SOCS2 ensures iTreg stability by limiting IL-4–mediated STAT6 activation and by preventing IL-13 and IFN-γ secretion. Importantly, it is now clear that Foxp3+ iTreg cells play a key role in establishing tolerance at mucosal surfaces and in regulating type 2 responses (32, 56, 57). Because SOCS2 is essential to ensure a stable expression of Foxp3 in iTregs and to inhibit Th2 differentiation (16), this work further emphasizes the importance of SOCS2 as an essential regulator of allergic immune responses.

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Disclosures

The authors have no financial conflicts of interest.
References


Supplementary Figure 1: Proliferation of CD4⁺Foxp3⁻ T cells.
CD4⁺CD25⁻ T cells were stimulated for 5 days with various concentration of TGF-b as described in Figure 4. Representative histogram showing CFSE dilution gated on WT or $Socs2^{-/-}$ CD4⁺Foxp3⁻ cells (A) or WT and $Socs2^{-/-}$ CD4⁺Foxp3⁻ cells overlaid (B).
Supplementary Figure 2: Suppressive function of WT and SOCS2 deficient iTregs

Socs2\(^{-/-}\) and WT Foxp3\(^{+}\) iTregs were sorted based on eGFP expression as described in material and methods and their ability to suppress the proliferation of WT and Socs2\(^{-/-}\) T\(_{conv}\) cells at different ratios, was evaluated by Cell Trace Violet (CTV) dilution as described in Fig. 2. (A) Representative histograms of T\(_{conv}\) cell proliferation when co-cultured with different ratio of iTreg. (B) Graph showing the mean suppression from triplicates, at different ratios of WT iTregs on WT or Socs2\(^{-/-}\) T\(_{conv}\) and of Socs2\(^{-/-}\) iTregs on WT or Socs2\(^{-/-}\) T\(_{conv}\). This experiment was performed twice with similar results.
Supplementary Figure 3: Transfer of naïve CD4⁺CD25⁻CD45RB⁺ T cells into IL23a⁻/-Rag1⁻/- mice
Naïve CD4⁺CD25⁻CD45RB⁺ T cells were sorted from Foxp3⁺GFP and Socs2⁻/- xFoxp3⁺GFP and 4x10⁵ cells were intra-peritoneally injected into IL23a⁻/-Rag1⁻/- mice. 8 weeks post transfer, mice were sacrificed, lymphocytes were extracted from the colon and analyzed by flow cytometry. 6 mice were reconstituted with WT cells, and 5 with Socs2⁻/- cells.
Supplementary Figure 4: IL-2, IL2 and IL-6 do not affect Socs2-/- iTreg instability

WT and SOCS2 deficient iTregs were sorted from Socs2-/- x Foxp3-eGFP and Foxp3-eGFP mice, and further stimulated with plate bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml), in the presence of IL-2 (5 U/ml), IL-12 (50 ng/ml), IL-6 (50 U/ml). After 3 days, culture supernatants were collected while the cells were stained with an anti-CD4. iTreg (CD4+Foxp3+) numbers were evaluated based on eGFP expression. Representative density plots from triplicates of cells gated on CD4+ cells are shown.