

# Regulation of Foxp3<sup>+</sup> Inducible Regulatory T Cell Stability by SOCS2

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Suppressor of cytokine signaling (SOCS) proteins are key regulators of CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells upregulated Foxp3 following in vitro TGF- $\beta$  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<sup>+</sup> iTregs secreted elevated IFN- $\gamma$  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<sup>+</sup> iTreg instability. Foxp3<sup>+</sup> iTregs are key regulators of immune responses at mucosal surfaces; therefore, this dual role of SOCS2 in both Th2 and Foxp3<sup>+</sup> iTregs reinforces SOCS2 as a potential therapeutic target for Th2-biased diseases. *The Journal of Immunology*, 2013, 190: 3235–3245.

**A**cquisition of Foxp3 expression by T cells is an essential anti-inflammatory mechanism that prevents the development of autoimmunity and induces peripheral tolerance (1). In CD4<sup>+</sup> T cells, Foxp3 induction can occur either in the thymus for natural regulatory T cells (nTregs) (2) or in the periphery for inducible Tregs (iTregs) (3, 4). In both cases, generation of Foxp3 cells is driven by IL-2 (5, 6) and TGF- $\beta$  (3, 7), whereas proinflammatory cytokines such as IL-6 or IL-4 limit

Foxp3 induction (8–10). Thus, diverse cytokine signaling pathways play key roles in the fate of Foxp3<sup>+</sup> Treg.

Important regulators of cytokine signaling are the suppressor of cytokine signaling (SOCS) proteins that are induced by STAT proteins following cytokine stimulation (11). SOCS act in a classical negative-feedback loop and thereby prevent STAT activation through defined mechanisms such as competitive binding to phosphorylated residues on the receptor complex and/or targeting components of the signaling cascade for proteasomal degradation (11). Recent studies implicate SOCS proteins as likely candidates to control differentiation of immune cells and thus help to shape the type of inflammatory response (12, 13). Importantly, SOCS proteins appear to modulate CD4<sup>+</sup> T cell polarization (14); this is exemplified by the differentiation of Th2 cells being regulated by SOCS3 and SOCS2 (15, 16), whereas Th17 differentiation has been shown to be regulated by SOCS1 and SOCS3 (17, 18).

Several groups have recently shown that SOCS1 plays a key role in thymic Foxp3<sup>+</sup> Treg development, suppressive function, and also their stability in vivo (19–22). Strikingly, disruption of SOCS1 in Foxp3<sup>+</sup> T cells leads to the spontaneous development of immune-mediated pathologies, such as dermatitis (21), despite increased Treg numbers in both the thymus and the periphery (19, 20). SOCS1-deficient Foxp3<sup>+</sup> Tregs have unstable expression of Foxp3, spontaneous secretion of IFN- $\gamma$  and IL-17, and consequently fail to limit IFN- $\gamma$  secretion by CD4<sup>+</sup> and CD8<sup>+</sup> T cells and protect from colitis (22).

To date, various transcriptome approaches have identified SOCS2 as preferentially expressed in nTregs and iTregs (23–25); however, a precise role for SOCS2 in Foxp3<sup>+</sup> Treg function or development has yet to be fully investigated. Using SOCS2-deficient animals, we have demonstrated that SOCS2 is required for stable expression of Foxp3 in iTregs in vitro and in vivo.

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Abbreviations used in this article: CTV, CellTrace Violet; iTreg, inducible regulatory T cell; LN, lymph node; mLN, mesenteric lymph node; nTreg, natural regulatory T cell; rh, recombinant human; SOCS, suppressor of cytokine signaling; Tcon, conventional T cell; Treg, regulatory T cell; WT, wild-type.

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SOCS2-deficient iTregs secreted elevated IFN- $\gamma$  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<sup>+</sup> 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*<sup>-/-</sup> 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 *Foxp3*<sup>eGFP</sup> (B6-*FoxP3*<sup>tm1mal</sup>) 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. *Foxp3*<sup>eGFP</sup> and OT-II mice were subsequently crossed to *Socs2*<sup>-/-</sup> mice in-house to generate *Socs2*<sup>-/-</sup>  $\times$  *Foxp3*<sup>eGFP</sup> and *Socs2*<sup>-/-</sup>  $\times$  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- $\mu$ m nylon cell strainers (BD Falcon), centrifuged 10 min at 700  $\times$  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  $\times$  g, cells present at the 75% and 40% Percoll interface were removed and washed in 30 ml PBS-FCS 2%.

### Flow cytometry

Surface marker expression was assessed by flow cytometry using CD3, CD4, CD8, CD25, CD45RB, 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<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells were magnetically isolated by negative selection for CD4 and subsequent positive selection for CD25 (Miltenyi Biotec). CD4<sup>+</sup>Foxp3<sup>-</sup>, CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, and naive CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> were sorted from *Foxp3*<sup>eGFP</sup> and *Socs2*<sup>-/-</sup>  $\times$  *Foxp3*<sup>eGFP</sup> to >99% purity with a BD FACS Aria II (BD Biosciences) using anti-CD4, anti-CD45RB, and anti-CD25 Abs (eBioscience). iTregs were obtained from presorted CD4<sup>+</sup>Foxp3<sup>-</sup> lymphocytes, and stimulated for 3 d with plate-bound anti-CD3 (2  $\mu$ g/ml; clone 145-2C11; eBioscience), anti-CD28 (1  $\mu$ g/ml; clone 37.51; eBioscience), and TGF- $\beta$  (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  $\mu$ M CFSE or 5  $\mu$ M 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  $\times$  10<sup>6</sup> cells/ml in complete RPMI-1640 medium supplemented with 10% FCS, penicillin,

and streptomycin (1%; PAA Laboratories), L-glutamine (200 nM; PAA Laboratories), and 2-ME (50  $\mu$ M; Sigma-Aldrich). Cells were then stimulated with plate-coated anti-CD3 (2  $\mu$ g/ml; eBioscience) and anti-CD28 (1  $\mu$ g/ml; eBioscience). CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>-</sup> T cells with rhTGF- $\beta$  (1–3 ng/ml; R&D Systems) and, in some assays, supplemented with neutralizing IL-4 Ab (20  $\mu$ g/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 ng/ml; R&D Systems).

### Suppressive assay

CD4<sup>+</sup>Foxp3<sup>-</sup> conventional T cells (Tconv) were purified from Foxp3<sup>eGFP</sup> or *Socs2*<sup>-/-</sup>  $\times$  Foxp3<sup>eGFP</sup> and labeled with CTV, as described above. Foxp3<sup>+</sup> Tregs or Foxp3<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> T cells were also added to the assay. Cells were then stimulated with soluble CD3 (2  $\mu$ g/ml; eBioscience) and CD28 (1  $\mu$ g/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*<sup>-/-</sup>  $\times$  OT-II mice. CD4<sup>+</sup> T cells were enriched by negative selection using the EasySep mouse CD4<sup>+</sup> T cell enrichment kit (StemCell Technologies). Enriched CD4<sup>+</sup> T cells were subsequently stained with anti-CD25 and anti-CD45RB Abs (eBioscience) and sorted to >99% purity with an FACSARIA II (BD Biosciences). Cells were then washed in PBS, and up to 4  $\times$  10<sup>6</sup> 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  $\mu$ g anti-IL-4 mAb (clone 11B11; American Type Culture Collection) or IgG1 isotype control i.v. in CD45.1 congenic 24 h prior to adoptive transfer of OT-II or *Socs2*<sup>-/-</sup>  $\times$  OT-II naive T cells and every subsequent 24 h for 5 d during OVA feeding.

Transfer of naive CD4<sup>+</sup> T cell into *I23a*<sup>-/-</sup> *Rag1*<sup>-/-</sup> mice leads to spontaneous development of iTreg in vivo (28). As a second model of iTreg induction, CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup>CD45RB<sup>hi</sup> naive T cells were sorted from *Foxp3*<sup>eGFP</sup> or *Socs2*<sup>-/-</sup>  $\times$  *Foxp3*<sup>eGFP</sup> mice, and 4  $\times$  10<sup>5</sup> cells were injected i.p. into *I23a*<sup>-/-</sup> *Rag1*<sup>-/-</sup> 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), Na<sub>3</sub>VO<sub>4</sub> (1 mM), leupeptin (10  $\mu$ g/ml), aprotinin (10  $\mu$ g/ml), and PMSF (1 nM) 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  $\gamma$ -tubulin (Sigma-Aldrich). IL-4, IL-5, IL-13, and IFN- $\gamma$  secretion in cell-culture supernatants was analyzed by ELISA (R&D Systems), following the manufacturer's instructions.

### 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<sup>+</sup> Treg numbers

*Socs2*<sup>-/-</sup> mice present with normal CD4<sup>+</sup> T cell development (16, 31). Interestingly, several groups have since characterized the Foxp3<sup>+</sup> Treg transcriptome and revealed that SOCS2 is prefer-

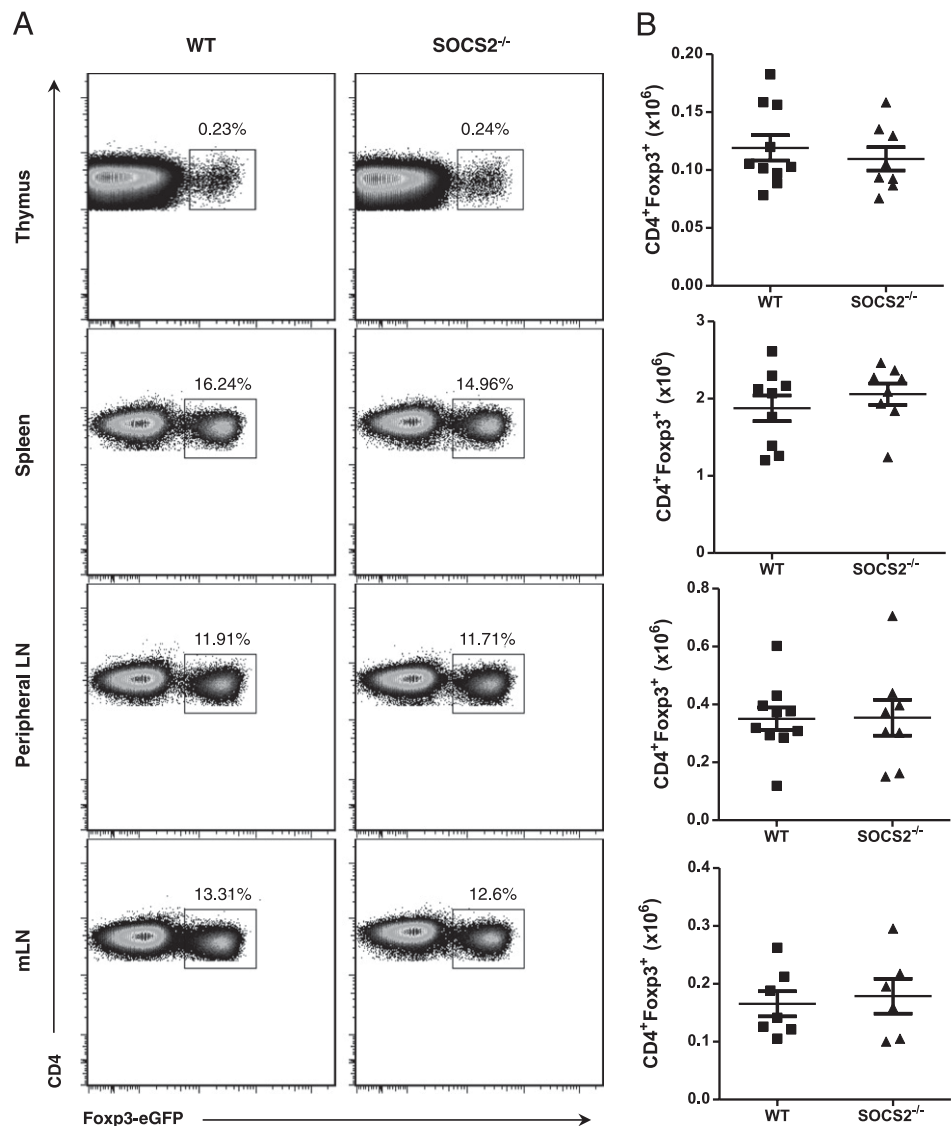
entially expressed in Foxp3<sup>+</sup> Treg cells compared with other T cell subsets, both in the thymus and in the periphery (23–25). We therefore investigated whether SOCS2 deficiency would affect Foxp3<sup>+</sup> nTreg development in vivo. However, following examination of the thymus, spleen, mLN, and other peripheral LN (axillary, brachial, and inguinal), similar frequencies (Fig. 1A) and absolute numbers (Fig. 1B) of CD4<sup>+</sup> Foxp3<sup>+</sup> T cells in Foxp3<sup>eGFP</sup> and Soc2<sup>-/-</sup> × Foxp3<sup>eGFP</sup> mice were found, suggesting that SOCS2 does not regulate Foxp3<sup>+</sup> Treg development under steady-state conditions.

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

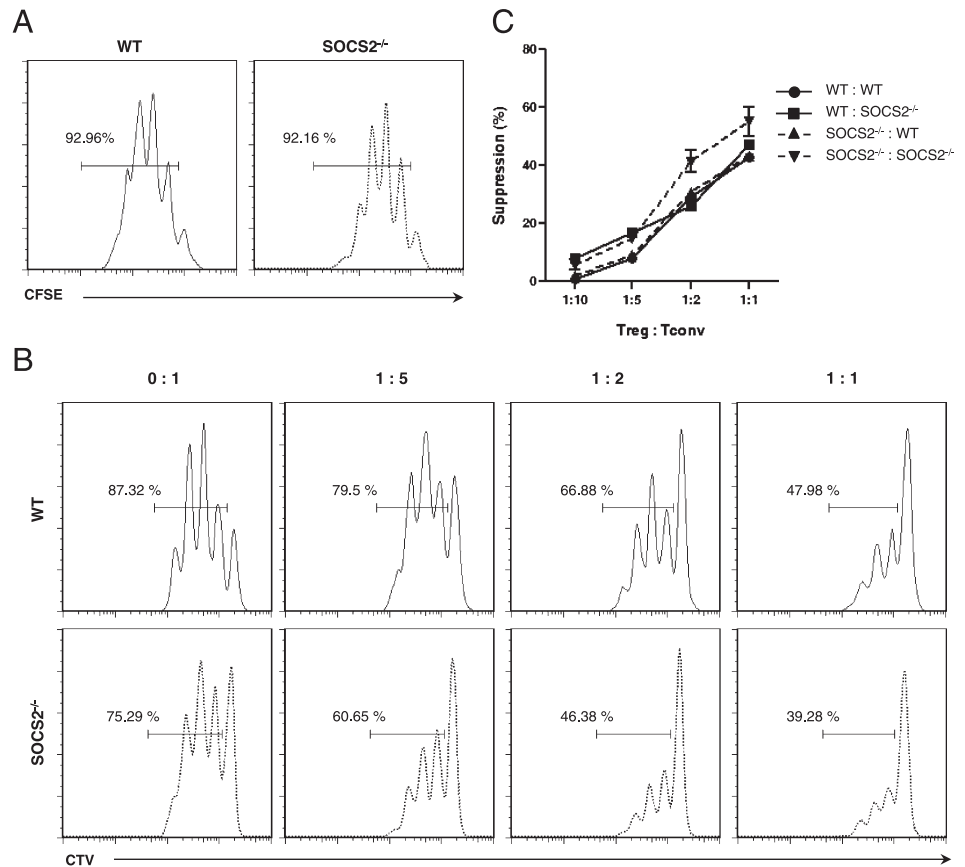
Sugimoto and colleagues (23) previously showed that CD4<sup>+</sup> CD25<sup>-</sup> T cells retrovirally transduced with SOCS2 became anergic and exerted some suppressive function, suggesting that these cells acquired a Treg-like phenotype. We thus examined whether SOCS2 deficiency would affect Treg cell division and their ability to inhibit CD4<sup>+</sup>CD25<sup>-</sup> T cell proliferation. Peripheral Tregs were purified by magnetic selection from wild-type (WT) C57BL/6J and Soc2<sup>-/-</sup> 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 Soc2<sup>-/-</sup> Foxp3<sup>+</sup>CD4<sup>+</sup> T cells strongly proliferated, and the

frequency of dividing SOCS2-deficient CD4<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup>Foxp3<sup>-</sup> T cells cocultured with various ratios of WT or Soc2<sup>-/-</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> T cells. Addition of either WT or Soc2<sup>-/-</sup> Tregs inhibited CD4<sup>+</sup>Foxp3<sup>-</sup> proliferation in a dose-dependent manner (Fig. 2B), with Soc2<sup>-/-</sup> Foxp3<sup>+</sup> 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<sup>+</sup>Foxp3<sup>-</sup> T cells more resistant or more susceptible to Treg suppression, we also examined the ability of Soc2<sup>-/-</sup> Tregs to inhibit WT CD4<sup>+</sup>Foxp3<sup>-</sup> T cell proliferation and of WT Tregs to suppress Soc2<sup>-/-</sup> CD4<sup>+</sup>Foxp3<sup>-</sup> T cells. In both cases, the suppression exerted was comparable to inhibition of WT CD4<sup>+</sup>Foxp3<sup>-</sup> 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<sup>+</sup> Tregs did not reveal any difference between WT and Soc2<sup>-/-</sup> cells (data not shown). Therefore, SOCS2-deficient Foxp3<sup>+</sup> Tregs presented with normal proliferation, suppressive function, and expression of Treg markers, highlighting that SOCS2 has a limited role in regulating Foxp3<sup>+</sup> Treg function and phenotype.

**FIGURE 1.** SOCS2 does not affect Foxp3<sup>+</sup> numbers under steady state. Lymphocytes were isolated from thymus, spleen, pooled peripheral axillary, brachial, and mLN from Soc2<sup>-/-</sup> × Foxp3<sup>eGFP</sup> and Foxp3<sup>eGFP</sup> mice, stained with anti-CD4, and analyzed by flow cytometry. (A) Representative dot plots of cells gated on CD4<sup>+</sup> lymphocytes. Indicated numbers represent the percent of CD4<sup>+</sup>Foxp3<sup>+</sup> cells within the CD4<sup>+</sup> population. (B) Graphs representing absolute numbers of CD4<sup>+</sup> Foxp3<sup>+</sup> cells in two groups of age- and sex-matched mice (WT *n* = 7–10; Soc2<sup>-/-</sup> *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<sup>+</sup> Treg phenotype. **(A)** CD4<sup>+</sup>CD25<sup>+</sup> lymphocytes purified with magnetic beads from WT and *Socs2*<sup>-/-</sup> 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<sup>+</sup>Foxp3<sup>+</sup> T cell proliferation was evaluated by CFSE dilution. Histograms gated on CD4<sup>+</sup>Foxp3<sup>+</sup> T cells are representative of triplicates, and the experiment was performed twice with similar results. **(B and C)** CD4<sup>+</sup>Foxp3<sup>+</sup> (Treg) and CD4<sup>+</sup>Foxp3<sup>-</sup> (Tconv) cells were sorted from *Socs2*<sup>-/-</sup> × *Foxp3*<sup>eGFP</sup> and *Foxp3*<sup>eGFP</sup> 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*<sup>-/-</sup> Tconv and of *Socs2*<sup>-/-</sup> Tregs on WT or *Socs2*<sup>-/-</sup> Tconv. Experiments were performed twice with similar results.



#### SOCS is highly expressed in Foxp3<sup>+</sup> iTreg cells

SOCS2 deficiency did not affect peripheral Foxp3<sup>+</sup> 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<sup>+</sup> iTregs generated in vitro compared with ex vivo peripheral Foxp3<sup>+</sup> Tregs. Therefore we examined SOCS2 protein levels in various CD4<sup>+</sup> subsets. As shown in Fig. 3, we could not detect SOCS2 protein expression in freshly isolated CD4<sup>+</sup> T cells or Foxp3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells. Purified WT and *Socs2*<sup>-/-</sup> CD4<sup>+</sup>CD25<sup>-</sup> 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<sup>+</sup> 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*<sup>-/-</sup> iTreg numbers were dramatically reduced (Fig. 4A, 4B;  $p < 0.01$ – $0.001$ ) compared with WT CD4<sup>+</sup> T cells that had maintained Foxp3 expression. Therefore, *Socs2*<sup>-/-</sup> CD4<sup>+</sup> T cells can efficiently induce Foxp3, but they are unable to maintain Foxp3 expression. Furthermore, although in-

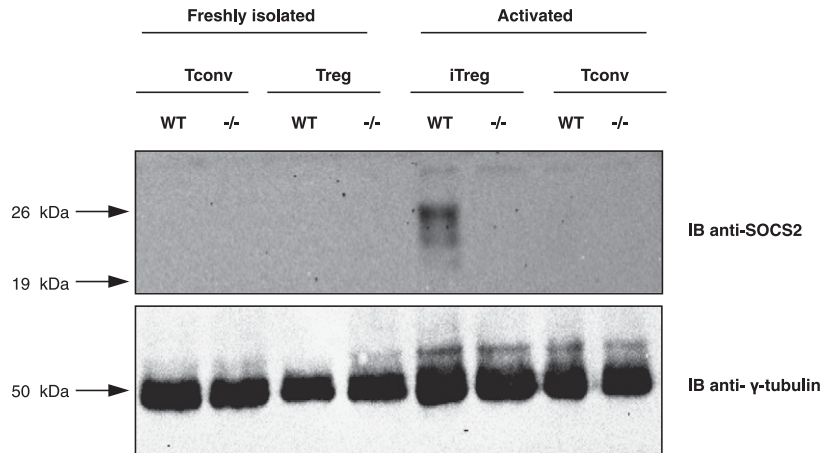
creased concentrations of TGF-β could partially rescue the loss of Foxp3 expression, iTreg numbers were still significantly lower in the absence of SOCS2 compared with WT controls (Fig. 4B).

We consequently examined the cytokine secretion profile of WT and *Socs2*<sup>-/-</sup> CD4<sup>+</sup>CD25<sup>-</sup> 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*<sup>-/-</sup> CD4<sup>+</sup>Foxp3<sup>-</sup> T cells as shown by CFSE dilution (Supplemental Fig. 1) and suggested that *Socs2*<sup>-/-</sup> iTregs might have impaired suppressive function. Indeed, failure to inhibit CD4<sup>+</sup>Foxp3<sup>-</sup> T cell proliferation would result in a relative decrease of Foxp3<sup>+</sup> iTreg frequency, whereas increased secretion of inflammatory cytokines would prevent further induction of Foxp3. However, examination of *Socs2*<sup>-/-</sup> iTreg suppressive function in vitro did not reveal any difference with WT iTregs at all ratios examined (Supplemental Fig. 2), suggesting that although *Socs2*<sup>-/-</sup> 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*<sup>-/-</sup> Foxp3<sup>+</sup> iTregs. Therefore, we believe that in the absence of SOCS2, the increased proliferation of CD4<sup>+</sup>Foxp3<sup>-</sup> 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). Naive CD4<sup>+</sup> CD45.2<sup>+</sup> T cells purified from *Socs2*<sup>-/-</sup> × OT-II 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<sup>+</sup>Foxp3<sup>+</sup> cells in the small intestine, mLN, and spleen was

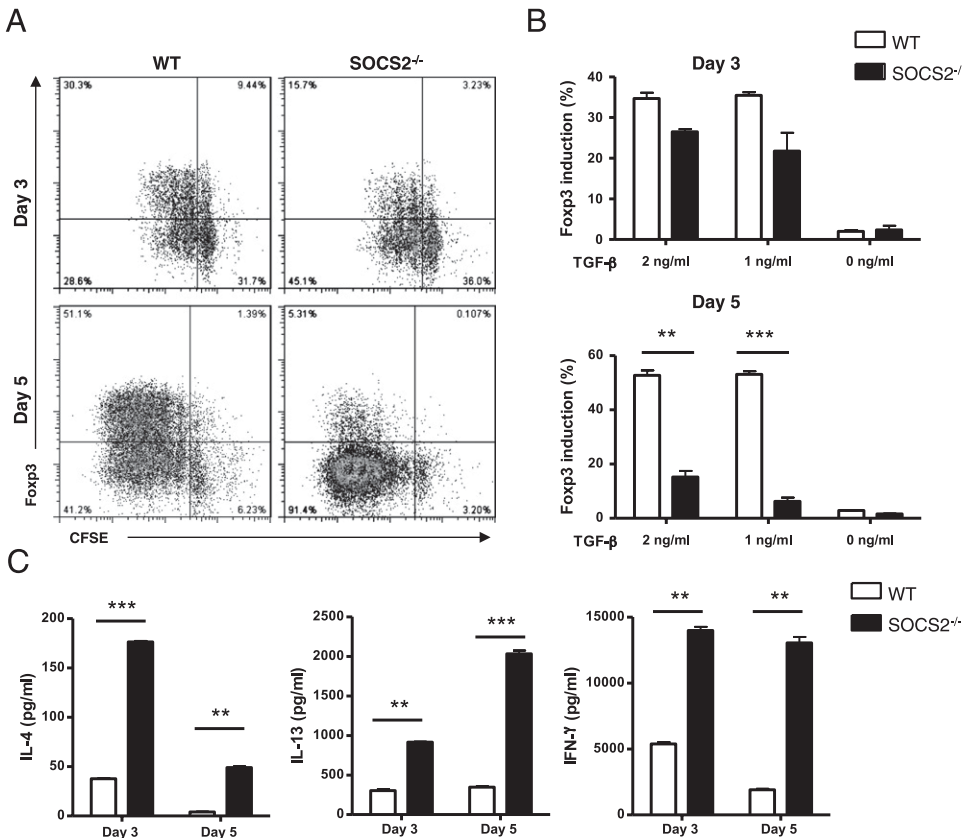
**FIGURE 3.** SOCS2 is highly expressed in iTreg. Freshly isolated Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup>) and Tconvs (CD4<sup>+</sup>Foxp3<sup>-</sup>) were sorted from *Socs2*<sup>-/-</sup> × *Foxp3*<sup>eGFP</sup> and *Foxp3*<sup>eGFP</sup> mice and directly lysed. iTregs were generated and sorted as described in the *Materials and Methods*, whereas CD4<sup>+</sup>Foxp3<sup>-</sup> 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.



evaluated. The transferred cells were identified based on CD45.2 expression (CD4<sup>+</sup>CD45.2<sup>+</sup>CD45.1<sup>-</sup>), 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<sup>+</sup> 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<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> sorted from WT and *Socs2*<sup>-/-</sup> mice were transferred into *Il23a*<sup>-/-</sup>*Rag1*<sup>-/-</sup> mice that lack IL-23 (35). Indeed, *Il23a*<sup>-/-</sup>*Rag1*<sup>-/-</sup> 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 *Socs2*<sup>-/-</sup> but not WT CD4<sup>+</sup> T cells induced weight loss in *Il23a*<sup>-/-</sup>*Rag1*<sup>-/-</sup> mice, suggesting that the reduced number of *Socs2*<sup>-/-</sup> 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<sup>+</sup> T cells upregulated Foxp3 following transfer into *Il23a*<sup>-/-</sup>*Rag1*<sup>-/-</sup> mice, whereas significantly less *Socs2*<sup>-/-</sup> CD4<sup>+</sup> 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.

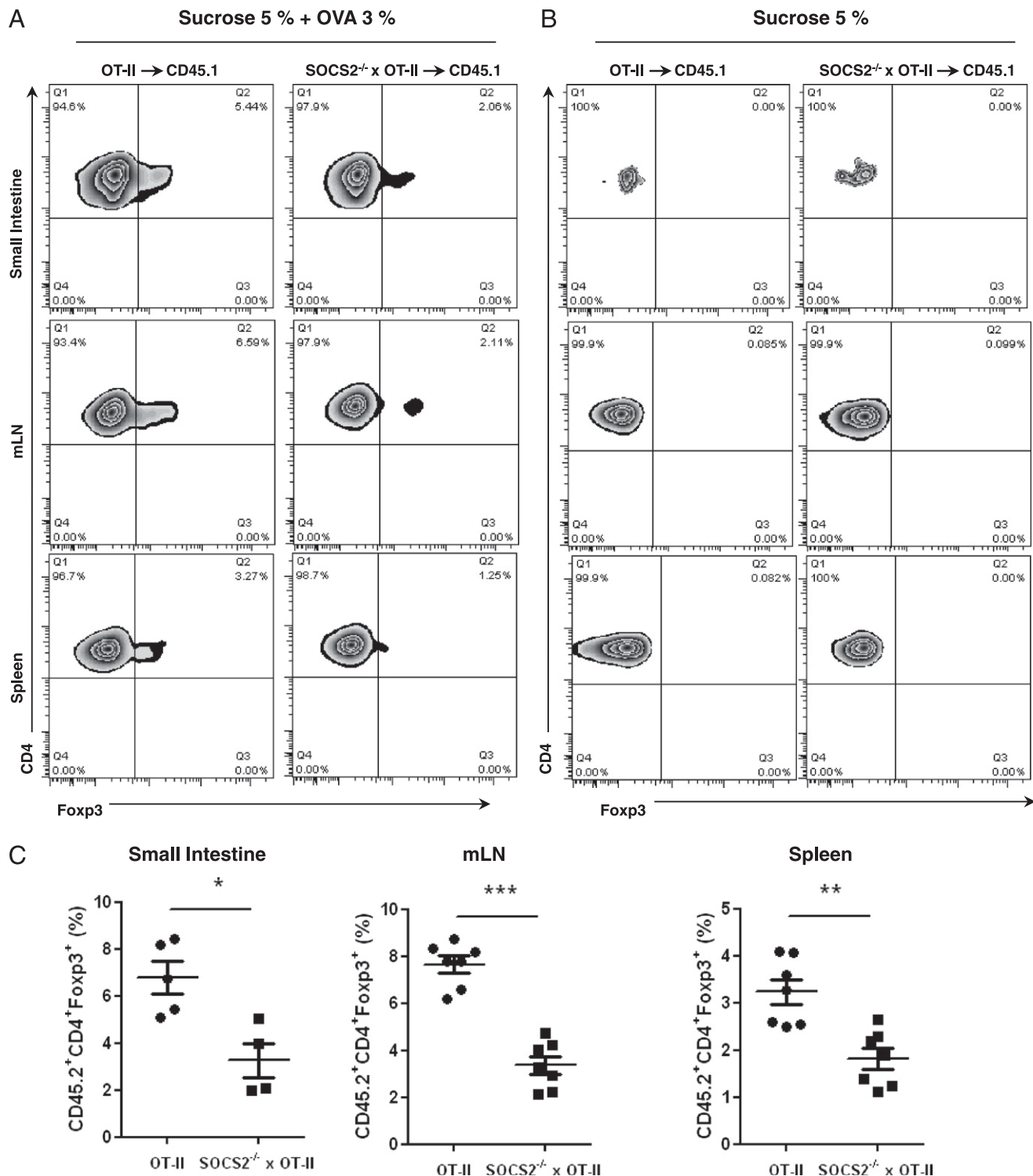


**FIGURE 4.** SOCS2-deficient Foxp3<sup>+</sup> iTregs fail to maintain Foxp3 expression in vitro. CD4<sup>+</sup>CD25<sup>-</sup> cells were purified with magnetic beads from lymphocytes extracted from *Socs2*<sup>-/-</sup> and WT mice. The cells were then labeled with CFSE and stimulated with plate-bound 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<sup>+</sup> 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<sup>+</sup> cell within CD4<sup>+</sup> population of CD4<sup>+</sup>CD25<sup>-</sup> 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<sup>+</sup>CD25<sup>-</sup> 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<sup>-/-</sup> iTreg instability*

SOCS2 clearly affects iTreg stability, and, based on our in vitro results (Fig. 4A, 4B), we hypothesized that *Socs2<sup>-/-</sup>* 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<sup>+</sup> Tregs can actively secrete proinflammatory cytokines, such as IFN- $\gamma$  or IL-17 (38), and also lose Foxp3 expression (39). To address our hypothesis, we sorted in vitro-derived WT and *Socs2<sup>-/-</sup>* Foxp3<sup>+</sup> iTregs from Foxp3<sup>eGFP</sup>



**FIGURE 5.** SOCS2 deficiency affects iTreg numbers in vivo. Naive CD45.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> T cells were sorted from *Socs2<sup>-/-</sup>* × OT-II or OT-II mice, and  $4 \times 10^6$  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 representatives of Foxp3 expression gated on CD4<sup>+</sup>CD45.2<sup>+</sup>CD45.1<sup>-</sup> 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<sup>-/-</sup>* × 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*<sup>-/-</sup> 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*<sup>-/-</sup> iTregs toward a Th2 phenotype. Interestingly, *Socs2*<sup>-/-</sup> iTregs secreted constitutively higher IFN- $\gamma$  levels compared with WT iTregs, regardless of the stimulating cytokines used (Fig. 6D), suggesting that SOCS2 constitutively repressed IFN- $\gamma$  secretion.

To confirm an *in vitro* role of IL-4 in mediating *Socs2*<sup>-/-</sup> iTreg instability, we repeated TGF- $\beta$ -mediated Foxp3 induction in CD4<sup>+</sup>Foxp3<sup>-</sup> 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). Naive CD4<sup>+</sup> CD45.2<sup>+</sup> T cells purified from *Socs2*<sup>-/-</sup>  $\times$  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*<sup>-/-</sup>  $\times$  OT-II and OT-II CD4<sup>+</sup> naive 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*<sup>-/-</sup>  $\times$  OT-II T cells compared with the same cohort of animals treated with the isotype control, in which, again, iTreg instability was observed. *Socs2*<sup>-/-</sup>  $\times$  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 enhanced numbers of both OT-II and *Socs2*<sup>-/-</sup>  $\times$  OT-II iTregs compared with isotype treated animals, which was consistent with a previous publication (32).

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

#### *SOCS2 regulates IL-4-induced STAT6 activation in Foxp3<sup>+</sup> iTreg*

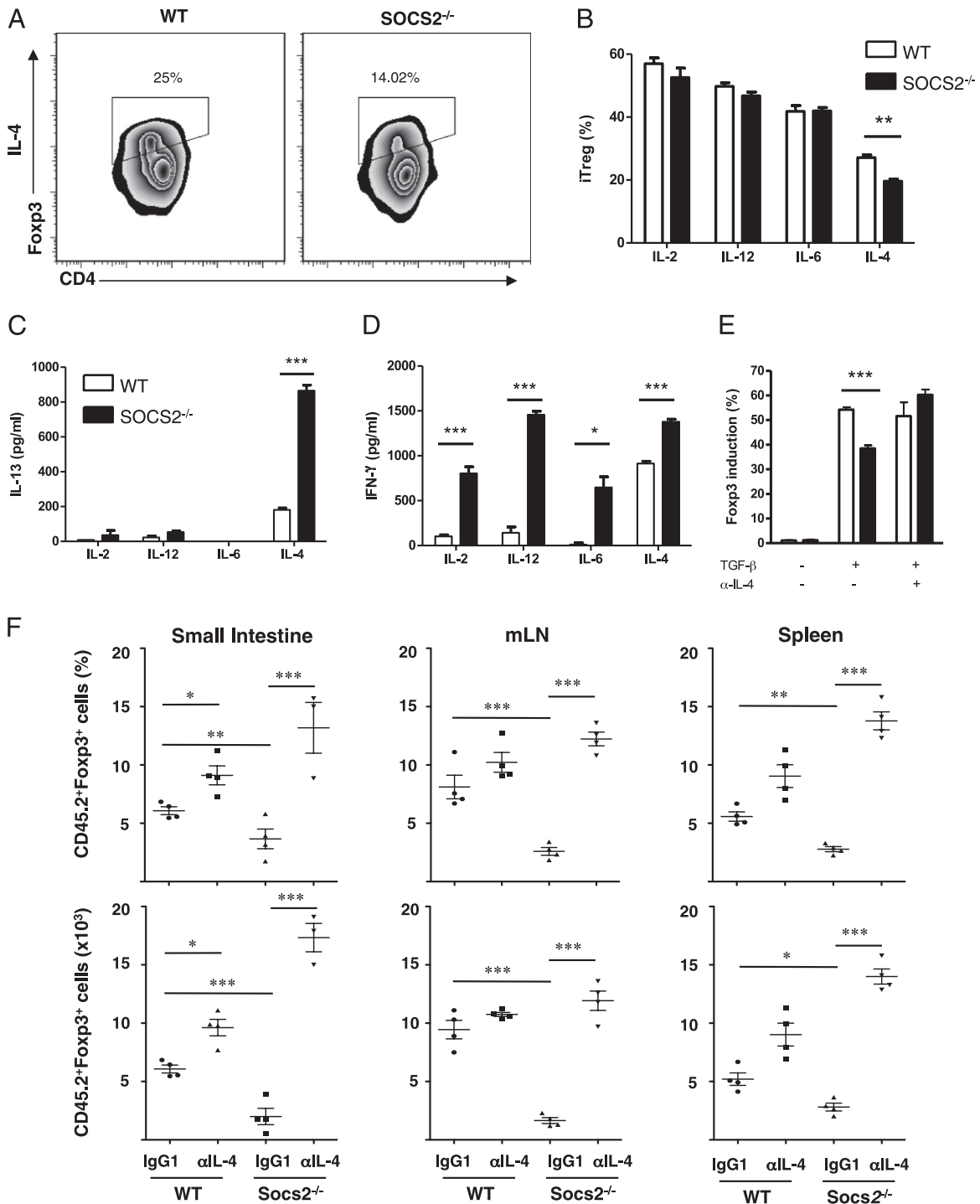
SOCS proteins are key regulators of STAT activation, and many studies have now shown that STATs play a key role in CD4<sup>+</sup> T cell polarization (40). In particular, STAT6 was found to bind to the Foxp3 promoter and to inhibit TGF- $\beta$ -mediated Foxp3 induction (9, 10, 41). Because *Socs2*<sup>-/-</sup> iTregs were more susceptible to IL-4-mediated Foxp3 instability, we hypothesized that SOCS2 might regulate IL-4 signaling in Foxp3<sup>+</sup> iTregs. We thus examined STAT6 activation following IL-4 stimulation in WT and *Socs2*<sup>-/-</sup> CD4<sup>+</sup> T cells prestimulated for 48 h with TGF- $\beta$ . 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*<sup>-/-</sup> CD4<sup>+</sup> T cells prestimulated with TGF- $\beta$ , demonstrating that SOCS2 prevents IL-4-induced Foxp3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> Treg development or function. However, we found that *Socs2*<sup>-/-</sup> mice displayed normal Foxp3<sup>+</sup> Treg numbers in the thymus and periphery under steady-state conditions and that SOCS2-deficient Foxp3<sup>+</sup> Tregs had a normal phenotype. Moreover, *ex vivo* Foxp3<sup>+</sup> Tregs purified from *Socs2*<sup>-/-</sup> mice exhibited normal proliferation and exerted similar suppressive function when compared with WT Foxp3<sup>+</sup> Tregs. This was consistent with the lack of SOCS2 protein expression in purified WT Foxp3<sup>+</sup> 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- $\beta$ , naive *Socs2*<sup>-/-</sup> CD4<sup>+</sup> T cells upregulated Foxp3 but failed to maintain its expression *in vitro*. *Socs2*<sup>-/-</sup> 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- $\gamma$ . Increased IFN- $\gamma$  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- $\gamma$  (42). Therefore, *Socs2*<sup>-/-</sup> iTregs could also be hyperresponsive to IL-2, and this may mediate the increased IFN- $\gamma$  secretion we observed (43, 44). Nevertheless, the enhanced IFN- $\gamma$  secretion by the *Socs2*<sup>-/-</sup> iTreg did not affect their stability, and therefore, we propose that IL-4 is the main cause of *Socs2*<sup>-/-</sup> 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- $\gamma$  by *Socs2*<sup>-/-</sup> 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 naive CD4<sup>+</sup> T cells isolated from *Socs2*<sup>-/-</sup>  $\times$  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*<sup>-/-</sup> mice do not develop any spontaneous autoimmune disorders (26), likely due to the fact that the pool of peripheral Foxp3<sup>+</sup> Tregs, that includes natural Tregs, is not affected by SOCS2 deficiency. Moreover, *Socs2*<sup>-/-</sup> 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<sup>+</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice succumb to *Toxoplasma gondii* infection (45) and are more susceptible to type 2 allergic responses (16). Although other immune cells mediate



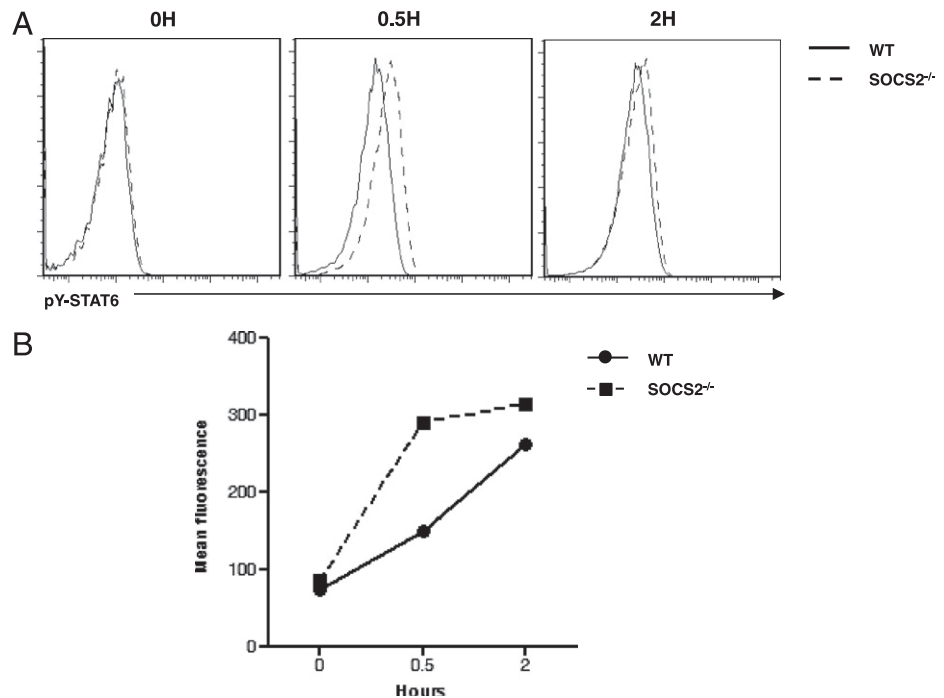
**FIGURE 6.** IL-4 promotes *Sox2*<sup>-/-</sup> iTreg instability. WT and SOCS2-deficient iTregs were sorted from *Sox2*<sup>-/-</sup> × *Foxp3*<sup>3eGFP</sup> and *Foxp3*<sup>3eGFP</sup> mice and further stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) in the presence of IL-2 (5 U/ml), IL-12 (50 ng/ml), IL-6 (50 U/ml), or IL-4 (5 ng/ml). After 3 d, culture supernatants were collected, whereas the cells were stained with anti-CD4. iTreg (CD4<sup>+</sup>Foxp3<sup>+</sup>) numbers were evaluated based on eGFP expression. **(A)** Representative density plot from triplicates of cells gated on CD4<sup>+</sup> cells (also see Supplemental Fig. 5). Frequency of iTregs are represented in the graph shown in **(B)**. IL-13 **(C)** and IFN-γ **(D)** secretion levels in supernatants collected from **(A)** and **(B)** were measured by ELISA. **(E)** CD4<sup>+</sup>Foxp3<sup>-</sup> T cells sorted from *Sox2*<sup>-/-</sup> × *Foxp3*<sup>3eGFP</sup> and *Foxp3*<sup>3eGFP</sup> mice were stimulated in vitro with TGF-β (1 ng/ml) with or without neutralizing IL-4 Ab (20 μg/ml). Levels of Foxp3 induction were evaluated at day 5 based on eGFP expression. **(F)** Total of 4 × 10<sup>6</sup> naive CD45.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> T cells were sorted from OT-II or *SOCS2*<sup>-/-</sup> × OT-II mice and adoptively transferred into CD45.1 transgenic mice by retro-orbital injection. Recipient mice received 3% OVA in drinking water for 5 d ± 0.25 mg IL-4 capture Ab or IgG1 isotype control i.v. every 24 h. Percentage (*top panel*) and absolute number (*bottom panel*) of CD45.2<sup>+</sup>Foxp3<sup>+</sup> from extracted small intestine, mLN, and spleen are presented. Data are representative of two experiments (four to five animals per group). Error bars represent 1 ± SEM. Statistical differences between WT and *Sox2*<sup>-/-</sup> were determined by the Student *t* test. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

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<sup>+</sup> Tregs and Th2 cells (46). This



**FIGURE 7.** SOCS2 regulates IL-4 signaling in iTregs. Foxp3<sup>+</sup> T cells were isolated from *Socs2*<sup>-/-</sup> × Foxp3<sup>eGFP</sup> and Foxp3<sup>eGFP</sup> mice and cultured with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) in the presence of TGF-β (3 ng/ml) for 48 h. The cells were then rested 2 h in serum-free RPMI-1640, and restimulated with IL-4 (200 ng/ml) for the indicated times. Phospho-STAT6 levels were examined by flow cytometry. **(A)** Representative histograms of WT and SOCS2-deficient CD4<sup>+</sup> cells stained with anti-pY-STAT6. **(B)** Graph representing the mean fluorescence of pY-STAT6. The experiment was done twice with similar results.



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<sup>+</sup> Treg function (47, 48), as GATA-3 inhibits Tbet and retinoic acid–related orphan receptor  $\gamma$ t expression (48), thus directly contributing to the stability of Foxp3<sup>+</sup> Treg phenotype. Furthermore, GATA-3–deficient Foxp3<sup>+</sup> Tregs 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<sup>+</sup> Tregs, and this might be directly controlled by STAT6. Indeed, IL-4 and IL-13 enhance GATA-3 expression levels in Th2 cells through STAT6 activation (50–52), but inhibit the development of iTregs (10, 41). 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell plasticity. Although controversial (36, 37), there is accumulating evidence that Foxp3<sup>+</sup> 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<sup>+</sup> Treg stability by limiting STAT1 activation (21, 22). Indeed *Socs1*<sup>-/-</sup> Foxp3<sup>+</sup> Tregs constitutively failed to maintain Foxp3 expression in vivo due to a hyperactivation of IFN-γ–mediated STAT1 (22). Moreover, SOCS1<sup>-/-</sup> 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<sup>+</sup> 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<sup>+</sup> Treg cells and plasticity of CD4<sup>+</sup> 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<sup>+</sup> 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.

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