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SOCS3 Deletion in T Lymphocytes Suppresses Development of Chronic Ocular Inflammation via Upregulation of CTLA-4 and Expansion of Regulatory T Cells

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Suppressors of cytokine signaling (SOCS) proteins are negative-feedback regulators of the JAK/STAT pathway, and SOCS3 contributes to host immunity by regulating the intensity and duration of cytokine signals and inflammatory responses. Mice with Socs3 deletion in myeloid cells exhibit enhanced STAT3 signaling, expansion of Th1 and Th17 cells, and develop severe experimental autoimmune encephalomyelitis. Interestingly, development of the unique IL-17/IFN-γ double-producing (Th17/IFN-γ and Tc17/IFN-γ) subsets that exhibit strong cytotoxic activities and are associated with pathogenesis of several autoimmune diseases has recently been shown to depend on epigenetic suppression of SOCS3 expression, further suggesting involvement of SOCS3 in autoimmunity and tumor immunity. In this study, we generated mice with Socs3 deletion in the CD4 T cell compartment (CD4-SOCS3 knockout [KO]) to determine in vivo effects of the loss of Socs3 in the T cell–mediated autoimmune disease, experimental autoimmune uveitis (EAU). In contrast to the exacerbation of experimental autoimmune encephalomyelitis in myeloid-specific SOCS3-deleted mice, CD4-SOCS3KO mice were protected from acute and chronic uveitis. Protection from EAU correlated with enhanced expression of CTLA-4 and expansion of IL-10–producing regulatory T cells with augmented suppressive activities. We further show that SOCS3 interacts with CTLA-4 and negatively regulates CTLA-4 levels in T cells, providing a mechanistic explanation for the expansion of regulatory T cells in CD4-SOCS3 during EAU. Contrary to in vitro epigenetic studies, Th17/IFN-γ and Tc17/IFN-γ populations were markedly reduced in CD4-SOCS3KO, suggesting that SOCS3 promotes expansion of the Th17/IFN-γ subset associated with development of severe uveitis. Thus, SOCS3 is a potential therapeutic target in uveitis and other autoinflammatory diseases. The Journal of Immunology, 2013, 191: 000–000.

The Jak/STAT pathway is an evolutionarily conserved signal transduction mechanism that regulates a myriad of physiological processes in mammals (1). The importance of regulating the initiation, duration, and intensity of STAT signals is underscored by the diverse array of pathologic conditions that arise from disruption or aberrant activation of STATs (2). Jak/STAT pathways are therefore under stringent regulation by a number of cytoplasmic proteins, including protein inhibitors of activated STAT, Src homology region 2 domain–containing phosphatase 1, SHIP-2, and the suppressors of cytokine signaling (SOCS) family of proteins. In the context of immune regulation or immune modulation therapy, much interest has focused on SOCS proteins, particularly SOCS1 and SOCS3 (3, 4). SOCS proteins are rapidly induced in response to cytokines (IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12, IL-21, IL-23, IL-27) or growth factors (ciliary neurotrophic factor, LIF, fibroblast growth factor, insulin-like growth factor-1, insulin), and their inhibitory effects derive from direct interaction with cytokine/growth factor receptors or signaling proteins, leading to proteosomal degradation of the receptor complex and termination of the signal.

Because of the relatively short half-life of SOCS proteins, their negative regulatory effects are generally transient. However, unabated stimulation of STAT signaling pathway by chronic inflammation can induce constitutive activation of SOCS expression (6). In some tissues this may result in persistent silencing of critical cellular pathways and predisposition to development of organ-specific diseases (7). SOCS proteins have now been implicated in diverse diseases, including autoimmune diseases, diabetes, and cancer (6–9).

SOCS3 regulates the activation and differentiation of naïve CD4 T cells, preferentially promoting Th2 and inhibiting Th1 differentiation via the inhibition of IL-12–mediated STAT4 activation (10, 11). It is constitutively expressed in naïve CD4+ T cells and its expression is inversely correlated with the level of IL-2 (11, 12). SOCS3 mediates the IL-27–induced suppression of CD28-mediated IL-2 production (13) and it blocks IL-2 production in response to TCR activation by suppressing calcineurin-dependent dephosphorylation and activation of NFATp (14). Unlike Th helper cells, regulatory T cells (Tregs) are deficient in SOCS3 protein expression, and in vitro overexpression of SOCS3 in Tregs decreased their proliferation and expression of Foxp3, suggesting that the SOCS3/IL-2 axis plays a critical role in controlling physiological levels of Tregs. In terms of the potential involvement of SOCS3 in autoimmune diseases, it has recently been shown that mice with Socs3 deletion in myeloid cells develop severe experimental autoimmune encephalomyelitis (EAE), suggesting that the STAT3/SOCS3 axis regulates neuroinflammation (15, 16). Alternatively, expression of SOCS3 in human arthritic chondrocytes contributes to cartilage damage during arthritis (17, 18). Interestingly, epigenetic suppression of SOCS3 expression in T cells promotes the expansion of a unique Tc17/IFN-γ double-producing CD8+ T cells implicated in several autoimmune diseases.
For propagation under the Th17 condition, the medium contained TGF-β were activated in plate-bound anti-CD3 Ab (3 μg/ml) and recorded. The clinical grading system for retinal inflammation was as lateral, and medial fields, and each individual lesion was identified, mapped, each eye by positioning the endoscope and viewing from superior, inferior, and anti-CD3, anti–IFN-γ, anti–IL-17, and anti-Foxp3 mAbs and corresponding isotype control Abs (BD Pharmingen) were performed on a FACS Calibur or LSR II (BD Biosciences) as previously described (31).

Cytokine analysis
WT or CD4−SOCS3KO T cells were activated for 4 d with anti-CD3/CD28 Abs, and multiplex ELISA of supernatants for cytokine secretion was performed using a commercial ELISA kit (R&D Systems, Minneapolis, MN).

Lymphocyte proliferation assay
Naïve T cells or LN cells from IRBP-immunized mice were cultured for 2–3 d in quintuplet cultures containing anti-CD3/CD28 Abs or IRBP. For the T cell suppression study, naïve T cells from WT or CD4−SOCS3KO mice were cultured with LN cells from WT mice with EAU for 3 d in medium containing IRBP. After 36 h, some cultures were pulsed with [3H]thymidine (0.5 μCi/10 μl/well) for 12 additional hours and analyzed. Data are mean cpm ± SE of responses of five replicate cultures.

Quantitative and semiquantitative RT-PCR analyses
Total RNA was extracted using the TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the procedures recommended by the manufacturer. All RNA samples were digested with RNase-free DNase I (Life Technologies) and purified by phenol/chloroform extractions. RNA integrity was verified by analysis of 18S and 28S rRNA expression on RNA gels. RNA (10 μg), SuperScript III reverse transcriptase (Life Technologies, Gaithersburg, MD), and oligo(dT)12–16 were used for first-strand synthesis as previously described (32). First-strand synthesis containing each mRNA sample but no reverse transcriptase was performed to control for possible DNA contamination; failure to obtain RT-PCR products with any of the PCR amplifiers confirmed the absence of DNA templates. All cDNA preparations were used as suitable substrates for PCR amplification on the basis of efficient amplification of a β-actin sequence. Real-time PCR was performed on an ABI 7500 (Applied Biosystems), and PCR parameters were as recommended for the TaqMan Universal PCR kit (Applied Biosystems). Primers and probes were purchased from Applied Biosystems.

Western blotting analysis
Preparation of whole-cell lysates and performance of Western blot analysis were as described (10). Cell extracts (20–40 μg/lane) were fractionated on 4–12% gradient SDS-PAGE, and Abs used were pSTAT3 (Cell Signaling Technology, CA),-D-1-thiogalactopyranoside (IPTG) and analyzed by SDS-PAGE, Coomassie blue staining, and Western blotting. GST or GST-SOCS3 fusion protein purified by glutathione-Sepharose 4B affinity was used for protein pull-down assays. Protein extracts (500 μg) isolated from activated CD4+ T cells were incubated with the purified GST-SOCS3 protein for 1 h at 4°C, washed twice, and the precipitates were analyzed by immunoblotting with CTLA-4 Abs.

Statistical analysis
Statistical analyses were performed by an independent two-tailed Student t test. Data are presented as means ± SD. A p value <0.05 was considered statistically significant.
Results

Generation and functional characterization of CD4-SOCS3 mice

We generated mice with targeted deletion of SOCS3 in the CD4 compartment (CD4-SOCS3KO) to investigate the function of SOCS3 in T cells during an autoimmune disease. PCR analysis of tail DNA of mice from the cross between CD4-Cre and Socs3fl/fl mouse strains on C57BL/6 background confirmed the generation of CD4-Cre/Socs3fl/fl double-positive mice (Fig. 1A). Expression of the Cre recombine under the CD4 promoter element (CD4-Cre strain) is initiated at the CD4/CD8 double-positive stage of the developing T cell, resulting in targeted expression the Cre protein in both CD4+ and CD8+ T cells (33, 34). We therefore expected that the expression of Cre at the double-positive stage would mediate SOCS3-specific deletion in both CD4+ and CD8+ T cells of the CD4-SOCS3KO mouse strain.

To confirm that SOCS3 was indeed deleted in CD4+ and CD8+ T cells of the CD4-SOCS3KO T cells, we isolated CD4+ and CD8+ T cells from WT and CD4-SOCS3KO mice and purified the cells by sorting. We prepared cDNA from RNA isolated from the sorted CD4+ or CD8+ T cells, and RT-PCR and quantitative RT-PCR analyses revealed that SOCS3 was deleted from CD4+ and also CD8+ T cells (Fig. 1B). Analysis of whole-cell extracts prepared from the sorted CD4+ T cells by Western blot assay confirmed specific deletion of the SOCS3 protein from the CD4 compartment (Fig. 1C). Western blot analyses further showed that SOCS3 is constitutively expressed in naive CD4+ T cells as previously reported (10), and we detected constitutive expression of pSTAT3 in CD4-SOCS3KO T cells but not in WT or hemizygous (CD4–/Socs3+/−)CD4+ T cells (Fig. 1D). To verify that functional SOCS3 function is lost in CD4-SOCS3KO T cells, we examined whether the CD4-SOCS3KO T cells had lost capacity to terminate STAT signals. Naive CD4+ T cells from WT or CD4-SOCS3KO mice were stimulated with anti-CD3/CD28 Abs for 3 d. The cells were then washed, starved in serum-free medium for 3 h on ice, and stimulated in medium containing IL-6 as indicated. The protein extracts were analyzed by Western blotting (Fig. 1E) and showed that the pSTAT3 signal was inhibited after 120 min in WT but not CD4-SOCS3KO T cells, confirming complete loss SOCS3 function in the CD4-SOCS3KO mice.

SOCS3 deficiency promoted the expansion of IL-10–expressing Tregs

Previous studies suggested that SOCS3 is an essential negative regulator of IL-23 signaling, and because IL-23 mediates STAT3 phosphorylation and Th17 generation, it was suggested that SOCS3 might therefore constrain the generation of Th17 differentiation (35). We therefore next investigated whether loss of SOCS3 in T cells would promote the expansion of Th17 cells. Naive CD4+ T cells were isolated and stimulated under nonpolarizing conditions or to mature into Th17 by using the appropriate cytokine and Abs mixture. The cytokine expression profile of the cultured cells was assessed by intracellular staining assay whereas cytokine secretion was detected using ELISA. Contrary to prediction, we show in this study that the CD4+ T cells lacking Socs3 generated comparable frequency of IL-17–expressing Th17 cells as did WT T cells (Fig. 2A), suggesting that SOCS3 is not a critical regulator of Th17 polarization. In contrast, SOCS3 was found to be a major regulator of IL-10–expressing cells (Fig. 2A). These results were confirmed by our ELISA data showing high levels of secretion of IL-10 by CD4-SOCS3KO T cells whereas IL-17 secretion was comparable between the WT and SOCS3KO T cells (Fig. 2B). The high level of IL-10–expressing T cells appeared to have produced an overall antiproliferative condition in the CD4-SOCS3KO cultures characterized by a relatively low percentage of activated IL-2– and IFN-γ–expressing T cells (Fig. 2A). These results thus suggest that SOCS3 may function to promote inflammatory activities of T cells.

Socs3 deletion in T cells confers protection from EAU

To investigate the impact of deleting SOCS3 in T cells on development and severity of acute and chronic uveitis, we induced EAU in WT (Socs3+/+) and CD4-SOCS3KO mice by active immunization with IRBP in CFA and monitored the course of the disease for 90 d by fundoscopy. During EAU in mice, the first 28 d constitutes the acute phase of EAU, and disease activity is most prominent between days 12 and 22 (36). Fundus images taken at days 14 and 21 show that both strains developed acute uveitis. However, EAU in the immunized WT mice was characterized by the infiltration of massive numbers of inflammatory cells into the retina resulting in destruction of retinal cells, development of retinal folding, serous retinal detachment, vasculitis, retinitis, choroiditis, and vitritis, and these pathological features are hallmarks of severe acute uveitis. Although very few cells are detectable in the retina 28 d after immunization, the chronic phase ensues and it is associated with significant ocular pathology characterized by severe papillitis, vasculitis with cuffing, retinal degenerative changes, and choroidal neovascularization (28, 36, 37). Thus, the clinical scores shown in Fig. 3B are monophasic and reflect continued progression of ocular pathology that culminates in vision loss (28). In contrast to EAU in...
least three independent experiments.

SOCS3 inhibits the suppressive activity of Tregs

Tregs expressing the transcription factor Foxp3 and induced T regulatory type 1 cells that lack Foxp3 expression play crucial roles in maintaining immune homeostasis, and they both induce immunosuppressive functions that regulate effector T cell function through the production of IL-10 (38). We isolated naive CD4^+ T cells from the WT or CD4-SOCS3KO mice, purified the cells using anti-CD4 magnetic beads, stimulated them for 3 d with anti-CD3/CD28 in the presence of TGF-β1, and examined whether SOCS3 regulates the expansion of Foxp3^+ Tregs. The analysis of Foxp3^+CD25^+ Tregs by intracellular flow cytometry showed a tremendous expansion of this regulatory cell population in the CD4-SOCS3KO T cell cultures (Fig. 5A), suggesting that SOCS3 inhibits their expansion. We next examined whether the IL-10–producing CD4-SOCS3KO T cells possess T cell–suppressive activities. We induced EAU in WT mice and on day 21 post-immunization and harvested cells from the LNs of the mice with EAU. The freshly isolated draining LN cells were then cocultured with naive CD4^+ T cells from either WT or CD4-SOCS3KO mice (ratios of naive CD4^+ T cells/uveitogenic LN cells are indicated). The proliferation of the uveitogenic T cells in response to re-stimulation with IRBP was assessed by a [3H]thymidine incorporation assay. In the first series of experiments performed at the 1:1 ratio of naive CD4^+ T cells/uveitogenic LN cells, SOCS3KO CD4 T cells did not appreciably alter the baseline proliferation, and the observed differences between the cultures containing WT or SOCS3KO can be attributed to background stimulation by WT cells versus no stimulation by SOCS3KO (Fig. 5B, left panel). However, in the next series of experiments performed at the 1:4 ratio (naive CD4^+ T cells/uveitogenic T cells), we observed significant suppression of proliferation in cultures containing CD4-SOCS3KO T cells whereas the WT T cells did not inhibit proliferation of the IRBP-stimulated uveitogenic LN cells (Fig. 5B, middle panel). Alternatively, the inhibitory effect of the CD4-SOCS3KO T cells was lost in experiments performed at the 1:16 ratio (naive CD4^+ T cells/uveitogenic T cells), and this can be attributed to the relatively low frequency of inhibitory CD4-SOCS3KO T cells in the cultures (Fig. 5B, right panel). Taken together, these results suggest that the suppressive activities of the CD4-SOCS3KO T cells might have contributed to the suppression of EAU in the CD4-SOCS3KO mice.

Socs3 interacts with CTLA-4 and regulates its steady-state level in CD4^+ T cells

It was previously reported that SOCS3 prevents excessive CD28-mediated IL-2 production by interacting with the CD28 costimulatory receptor and targeting it for proteosomal degradation (11). This report also indicated that the association between CD28 and SOCS3 required the interaction of SOCS3 with the tyrosine phosphorylation of a motif in the cytoplasmic region of CD28 (11). This report also indicated that the association between CD28 and SOCS3 required the interaction of SOCS3 with the tyrosine phosphorylation of a motif in the cytoplasmic region of CD28 (11).

**FIGURE 2.** SOCS3-deficient CD4^+ T cells promote the expansion of IL-10–producing cells. Naïve CD4^+ T cells isolated from WT and CD4-SOCS3KO mice were stimulated with anti-CD3/CD28 for 4 d under Th0 or Th17 polarizing conditions and then analyzed for cytokine expression by the intracellular cytokine staining assay (A) or for cytokine secretion by ELISA (B). Numbers in quadrants (A) indicate percentage of T cells expressing IL-2, IL-10, IL-17, or IFN-γ. Results are representative of at least three independent experiments.

**Resistance of CD4-SOCS3KO mice to EAU correlates with expansion of Tregs**

To investigate the mechanistic basis for the resistance of CD4-SOCS3KO mice to EAU, we analyzed the immune phenotype and cytokine expression profile of T cells in the peripheral blood of IRBP/CFA-immunized WT or SOCS3KO mice (day 21 post-immunization), as well as T cells from unimmunized C57BL/6 mice. Analysis of the expression of activation markers revealed that the frequency of activated CD4^+ T cells is much reduced in CD4-SOCS3KO blood compared with the WT (Fig. 4A), indicating that SOCS3 may promote an anti-inflammatory environment during EAU. Both Th1 and Th17 cells have been implicated in the etiology of human and mouse uveitis (25, 26), and SOCS3 is thought to be an essential negative regulator of IL-23 signaling and Th17 differentiation (35). Contrary to the expectation that Th17 cells would be aberrantly expanded in the CD4-SOCS3KO mice during EAU, we observed a slight decrease in the percentage of IL-17–expressing Th17 cells as well as a decrease in IFN-γ–expressing Th1 cells (Fig. 4B). Alternatively, we observed a higher frequency of IL-10–expressing cells in the blood of IRBP-immunized CD4-SOCS3KO compared with WT mice by FACS (Fig. 4C) and ELISA (Fig. 4D). The latter result may partially explain the lower numbers of activated cells and Th1 or Th17 in CD4-SOCS3KO mice. Of significant interest is the >3.5-fold reduction of IL-17/IFN-γ double-producing Th17 cells (Fig. 4B) associated with several inflammatory diseases, including EAU (19–22). Because SOCS3 is also deleted in CD8^+ T cells, we also examined whether development of the unique Tc17/IFN-γ CD8^+ population is also compromised in CD4-SOCS3KO mice. Similar to CD4^+ T cells, we observed a 4.4-fold reduction in the Tc17/IFN-γ CD8^+ population (Fig. 4E), and in contrast to CD4^+ T cells, the CD4-SOCS3KO mice have a higher percentage Tc17 cells (Fig. 4F).
extracts from the T cells showed an increase of CTLA-4 protein in the naive or activated CD4-SOCS3KO cells compared with WT T cells (Fig. 6A). Consistent with previous reports, we also found an increase of CD28 in the CD4-SOCS3KO T cells, suggesting that SOCS3 is a negative regulator of CTLA-4 and CD28. To investigate whether SOCS3 regulates the level of CTLA-4 in vivo, we analyzed the level of CTLA-4–expressing CD4+ T cells in the blood of IRBP/CFA-immunized WT or SOCS3KO mice (day 21 postimmunization). The frequency of CTLA-4–expressing T cells was doubled in CD4-SOCS3KO compared with WT mice (Fig. 6B), further supporting the observed augmented suppressive activity of Tregs generated from CD4-SOCS3KO mice (Fig. 5).

Although the reduced EAU severity in CD4-SOCS3KO mice is associated with increased CTLA-4 expression, more studies are required to firmly establish a causal relationship between Socs3 deletion, CTLA-4 increase, and suppression of uveitis. To directly examine whether SOCS3 interacts with CTLA-4, we generated a GST-SOCS3 fusion protein in a bacterial expression system. We characterized and purified the recombinant protein by affinity chromatography following induction with IPTG (Fig. 6C, 6D). The GST-SOCS3 protein was identified and verified by Western blotting (Fig. 6E). Pull-down protein assays demonstrated that SOCS3 binds to CTLA-4 in CD4 T cells (Fig. 6F).

Discussion

SOCS3 is constitutively expressed in naive T cells, and zero following TCR activation, the SOCS3 protein is rapidly downregulated with concomitant upregulation of IL-2 levels, leading to the suggestion that SOCS3 levels are inversely correlated with the amount of IL-2 secretion and proliferative responses of differentiating T helper cells (11, 12). In this study, we have shown that T cells that lack SOCS3 produce less IL-2 (Fig. 2A) and exhibit a less activated phenotype (Fig. 4A), and we have provided mechanistic insights into how the antagonism between SOCS3 and IL-2 contributes to the regulation of T cell activation/proliferation.

Socs3 deletion is embryonic lethal. Thus, mouse strains with tissue-specific Socs3 deletion have been generated by Cre-Lox technology as an approach to understand in vivo functions of Socs3. For example, the CreMMTV/Socs3fl/fl strain generated by mating Socs3fl/fl with mice expressing Cre under the control of the mouse mammary tumor virus (MMTV) promoter (CreMMTV), exhibited loss of >99% of Socs3 mRNA in thymocytes, lymphocytes, and activated CD4+ T cells (35). Consistent with our findings in the CD4-SOCS3KO mice, the CreMMTV/Socs3fl/fl mice had normal numbers of LN CD4 and CD8 cells. In vitro analysis of CD4+ T cells from the CreMMTV/Socs3fl/fl mice revealed that SOCS3 is an essential negative regulator of IL-23 signaling, the inhibition of which constrained the generation of Th17 differentiation (35). However, in vivo function of Socs3 deletion was not examined. In this study, we have used the CD4-SOCS3KO mice to examine the role of SOCS3 in an organ-specific autoimmune disease.

Analysis of the CD4-SOCS3KO mice revealed that SOCS3 is essential for the regulation of physiological levels of two essential T cell cosignaling receptors, CD28 (costimulatory receptor) and CTLA-4 (coinhibitory receptor), in naive and activated CD4+ T cells. CD28 is constitutively expressed on naive CD4+ and CD8+ T cells, and following ligation by B7-1 and B7-2 molecules on APCs, it provides essential costimulatory signals for IL-2 production, T cell proliferation, and survival. However, CD28 and CTLA-4 share the highly conserved MYYPYP motif through

FIGURE 3. Loss of SOCS3 in CD4+ T cells confers protection from uveitis. EAU was induced in WT or CD4-SOCS3KO mice by active immunization with IRBP in CFA. Progression and severity of EAU was monitored for 90 d by fundoscopy or histology. (A) Fundus images were taken using an otoendoscopic imaging system, and the development of papillitis (black arrows), retinal vasculitis (blue arrows), and inflammatory infiltrates (white arrows) are indicated. (B) Clinical score was based on changes at the optic nerve disc, retinal vessels, and surrounding tissues as described in Materials and Methods. ***p < 0.001, ****p < 0.0001. (C) Eyes were enucleated on day 21 postimmunization, fixed in paraffin, and stained with H&E. Infiltrated inflammatory cells in the vitreous (V) are denoted by black arrows; blue asterisks indicate retinal folds. Original magnifications are indicated in each panel. (D) Disease score is based on histology. **p < 0.01. CH, Choroid; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OpN, optic nerve; RPE, retinal pigmented epithelial layer.
which they competitively bind to B7-1 and B7-2 cells (39). Thus, following T cell activation, CTLA-4 expression is upregulated, resulting in the suppression of CD28-mediated T cell responses by outcompeting CD28 for binding to B7-1 and B7-2 cells. Although the level of CTLA-4 is relatively low in naive T cells, we observed substantial increase of CTLA-4 in naive T cells of the CD4-SOCS3KO mice (Fig. 6A). We also show that the activated T cells that lack SOCS3 maintained their relatively high levels of CD28 compared with WT (Fig. 6A). These results suggest that SOCS3 may serve a gatekeeper function in T cells. Alternatively, constitutive expression of SOCS3 in naive T cells would ensure maintenance of low levels of CTLA-4, allowing the cell to be poised to receive costimulatory CD28 signals for IL-2 production and growth. The increase in IL-2 production that follows T cell activation induces a transient diminution of SOCS3 level, as previously reported (12) and as shown in this study, and a decrease in SOCS3 promotes the elevation of CTLA-4 level (Fig. 6A, 6B). It has been suggested that following the TCR-mediated increase in CTLA-4 expression, CTLA-4 accumulates in the same region of the immunological synapse as CD28, physically excluding CD28 from the central supramolecular activation cluster and disrupting positive costimulatory signaling (40). In this study, we provide empirical evidence that SOCS3 indeed physically interacts with CTLA-4 and might therefore regulate the steady-state levels of CD28 and CTLA-4. Interestingly, CD4-SOCS3KO T cells continue to express high levels of CD28 after TCR activation (Fig. 6A), consistent with reports that SOCS3 regulates CD28 levels by physically interacting with and targeting CD28 for proteosome-mediated degradation (11). It is therefore intriguing that the dynamic regulation of steady-state levels of CD28 and CTLA-4 by SOCS3 might contribute to mechanisms that initiate T cell activation and those that eventually restore the activated T cell to its resting state.
Because of the mutual antagonism between IL-2 and SOCS3 and the fact that different T cell subsets require different amounts of IL-2 for differentiation, growth, or effector functions, we expected the loss of SOCS3 to confer selective advantages to some T cell subsets. Thus, the CD4−SOCS3KO T cells produced >3-fold fewer IL-2–expressing CD4+ T cells following TCR activation (Fig. 2A, top left panels), and this favored the expansion of IL-10–producing Tregs (Figs. 4C, 5B) at the expense of IL-17–producing Th17 cells (Fig. 4B) during EAU. In this context it is notable that SOCS1 and SOCS3 play essential roles in T cell development, function, and lineage commitment, and various CD4+ T cell subsets differ in the repertoire and amount of SOCS proteins they express (10). For example, all T helper cells express SOCS1 and SOCS3 proteins during their development, and constitutive expression of SOCS1 or SOCS3 promotes commitment and stability of the Th1 and Th2 lineages, respectively (10). In contrast, Tregs do not express SOCS3 protein, and in vitro overexpression of SOCS3 in Tregs decreased their proliferation, Foxp3 expression, and suppressive function (41). Also note that targeted degradation of SOCS1 is required to maintain competitive fitness of Tregs, underscoring the importance of stringent regulation of the levels of SOCS1 and SOCS3 proteins in Tregs (42, 43).

EAU is a CNS autoimmune disease that shares essential features with EAE, and these diseases serve as models for human uveitis and multiple sclerosis (24, 44). Studies utilizing these model diseases have provided valuable insights into pathogenic processes mediated by Th1 and Th17 cells in immune-privileged organs such as the eye and brain (24, 44). The role of Th1 and Th17 cells in both diseases is still controversial. However, there is emerging consensus that production of both IL-17 and IFN-γ is required for the pathology observed in these CNS autoimmune diseases (21, 26, 45). In EAU the earliest T cells detected in the blood or retina after disease induction are Th17 cells (21, 25). The highest level is detected at the peak of disease and rapidly declines upon disease resolution. Alternatively, Th1 cells are relatively low at early stages of EAU but steadily increase and become abundant at the resolution stage of the acute disease, leading to the notion that Th17 cells initiate uveitis whereas Th1 cells may be protective (21, 25). Of particular interest are reports showing that the Th17 subset is plastic and rapidly converts to Th1-like cells, accounting for the appearance of the IL-17/IFN-γ double-producing Th17 population in several diseases (19–22). The IL-17/IFN-γ double-producing Th17 cells accumulate in the blood and retina at the peak of EAU but disappear at resolution of the disease (21). Because both IFN-γ and IL-17 contribute to EAU pathology, we think that targeting this population would ameliorate uveitis. In this study, we have shown that loss of Socs3 in CD4+ or CD8+ T cells leads to dramatic reduction of the IL-17/IFN-γ double-producing Th17 population and correlates with resistance to EAU (Fig. 4B, 4D). It is also remarkable that during EAU, the main impact of the loss of Socs3 is on the level of IL-17 expression.

Uveitis is a potentially sight-threatening idiopathic disease often characterized by repeated cycles of remission and recurrent intraocular inflammation. This makes prolonged use of the standard therapy, steroids, problematic because of their serious adverse effects, including glaucoma and cataract. This concern has been the impetus for seeking alternative therapeutic approaches. CTLA-4 has been shown to limit the expansion of encephalitogenic T cells in EAE and mediates remission in relapsing EAE models (46–48). In this study, we have shown that targeting SOCS3 may be useful in modulating CTLA-4 levels and regulating Tregs and the IL-17/IFN-γ double-producing Th17 population, suggesting that further understanding of the role of the STAT3/SOCS3 axis during intraocular inflammation may lead to development of rational therapeutics for uveitis.

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**Disclosures**

The authors have no financial conflicts of interest.
SOCS3 INHIBITS CTLA-4 EXPRESSION AND Treg DEVELOPMENT

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