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TGF-β Promotes Th17 Cell Development through Inhibition of SOCS3[1]

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TGF-β, together with IL-6 and IL-21, promotes Th17 cell development. IL-6 and IL-21 induce activation of STAT3, which is crucial for Th17 cell differentiation, as well as the expression of suppressor of cytokine signaling (SOCS)3, a major negative feedback regulator of STAT3-activating cytokines that negatively regulates Th17 cells. However, it is still largely unclear how TGF-β regulates Th17 cell development and which TGF-β signaling pathway is involved in Th17 cell development. In this report, we demonstrate that TGF-β inhibits IL-6- and IL-21-induced SOCS3 expression, thus enhancing as well as prolonging STAT3 activation in naive $CD4^+CD25^-$ T cells. TGF-β inhibits IL-6-induced SOCS3 promoter activity in T cells. Also, SOCS3 small interfering RNA knockdown partially compensates for the action of TGF-β on Th17 cell development. In mice with a dominant-negative form of TGF-β receptor II and impaired TGF-β signaling, IL-6-induced $CD4^+$ T cell expression of SOCS3 is higher whereas STAT3 activation is lower compared with wild-type B6 $CD4^+$ T cells. The addition of a TGF-β receptor I kinase inhibitor that blocks Smad-dependent TGF-β signaling greatly, but not completely, abrogates the effect of TGF-β on Th17 cell differentiation. Our data indicate that inhibition of SOCS3 and, thus, enhancement of STAT3 activation is at least one of the mechanisms of TGF-β promotion of Th17 cell development. ***The Journal of Immunology,*** **2009, 183: 97–105.**

The T helper cells designated "Th17 cells" have been identified as a lineage distinct from Th1 and Th2 cells and are required for the induction of several autoimmune diseases, including collagen-induced arthritis, experimental autoimmune encephalitis (EAE),[3] and inflammatory bowel disease, and also for the ability to clear bacterial infections of the intestine and the airways (1–6). Th17 cells produce IL-17 (IL-17A), IL-17F, IL-21, and IL-22, all of which regulate inflammatory responses by different cells. There has been an intensive interest in defining how these pathogenic T cells develop and the factors that regulate their function. Similarly as Th1 and Th2 cells, Th17 cells require specific cytokines and transcription factors for their development. It has been shown that IL-6 and TGF-β initiate Th17 cell differentiation by conditioning naive T cells to become receptive to the additional cytokine signals required for differentiation toward the Th17 cell lineage through the induction of IL-21, which acts in a positive feedback loop to induce more IL-21 expression. IL-21, in turn, activates IL-23R expression in the presence of TGF-β. IL-23, like IL-6 and IL-21, can then synergize with TGF-β-initiated signals to induce IL-17 and other Th17 lineage cytokines to complete the development of Th17 cells. IL-6, IL-21, and IL-23 can all activate STAT3, and such activation of STAT3 is crucial for their effects on Th17 cell differentiation (7–12). Overexpression of a constitutively active STAT3 in T cells promotes Th17 cell differentiation, whereas STAT3 deficiency impairs Th17 cell differentiation both in vitro and in vivo (9, 13). STAT3 regulates expression of the Th17 cell-specific transcription factors RORγt and RORα (where ROR is retinoic acid-related orphan receptor), key transcription factors for Th17 cell differentiation (14). Conversely, IFN-γ, IL-2, IL-4, and IL-27 negatively regulate Th17 cell development (2, 3, 15–17).

Although Th17 cell differentiation requires TGF-β at every stage, it is still largely unknown how TGF-β acts on Th17 cell differentiation and which TGF-β signaling pathway is involved in Th17 cell development. In recent reports, it has been shown that TGF-β induces both Foxp3 and RORγt expression and that Foxp3 inhibits RORγt function (18, 19). IL-6 or IL-21 itself also induces low levels of RORγt expression, and TGF-β greatly enhances such RORγt expression (20). IL-6 not only induces STAT3 activation in T cells, it also stimulates suppressor of cytokine signaling (SOCS)3 expression, a major negative feedback regulator of STAT3-activating cytokines (21, 22). SOCS3 negatively regulates Th17 cell development, in that SOCS3 deficiency in T cells results in higher levels of Th17 cells both in vitro and in vivo (23). In contrast, overexpression of SOCS3 in T cells results in reduced STAT3 phosphorylation, much less Th17 cell development, and

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[3] Abbreviations used in this paper used: EAE, experimental autoimmune encephalitis; DN, dominant negative; ROR, retinoic acid-related orphan receptor; RPA, RNase protection assay; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; TGF-βR, TGF-β receptor; Treg, regulatory T cell; WT, wild type.

significantly delayed EAE onset (24). In this report, we investigated whether TGF-β promotes Th17 cells through the inhibition of SOCS3 and, thus, the enhancement of STAT3 activation. We report here that TGF-β inhibited IL-6- and IL-21-induced SOCS3 through inhibition of SOCS3 promoter activity, thus enhancing as well as prolonging STAT3 activation in naive $CD4^+CD25^-$ T cells. In mice with a dominant-negative (DN) form of TGF-β receptor (TGF-βR) type II (TGF-βRII) and impaired TGF-β signaling, IL-6-induced $CD4^+$ T cell expression of SOCS3 was higher whereas STAT3 activation was lower compared with wild-type (WT) B6 $CD4^+$ T cells. Consistent with these results, there were fewer Th17 cells in the intestinal lamina propria of TGF-βRII DN mice compared with WT mice. The addition of a TGF-βR type I (TGF-βRI) kinase inhibitor that blocks Smad-dependent TGF-β signaling inhibited the effect of TGF-β on Th17 cell differentiation.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory. C57BL/6 TGF-βRII DN transgenic mice were bred in the Animal Facility at the University of Alabama at Birmingham (Birmingham, AL). All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Antibody and reagents

Recombinant murine IL-6, TGF-β1, and IL-21 were from R&D Systems. Abs against phospho-$STAT3^{Tyr705}$ and phospho-$STAT3^{Ser727}$ were from Cell Signaling Technology. Abs against STAT3 and actin were from Santa Cruz Biotechnology. The Smad inhibitor SB505124 was from Calbiochem Biochemicals.

Isolation of $CD4^+$ T cells and $CD4^+CD25^-$ T cells

$CD4^+$ T cells were isolated using anti-mouse CD4-magnetic beads (BD Biosciences). Briefly, spleen and mesenteric lymph node cells were washed twice and incubated with anti-CD4 beads at 4°C for 30 min and then separated by magnetic field. When checked by flow cytometry, >95% of the cells were $CD4^+$ T cells. $CD4^+CD25^-$ T cells were isolated using the $CD4^+CD25^-$ T cell isolation kit from Miltenyi Biotec based on the instructions provided by the manufacturer.

RNA isolation, riboprobes, RNase protection assay (RPA), and RT-PCR

Total cellular RNA was isolated from unstimulated, IL-6-, IL-21-, IL-6 plus TGF-β1-, or IL-21 plus TGF-β1-treated $CD4^+$ T cells. Riboprobes for murine SOCS3 and GAPDH were hybridized with 5 μg of total RNA and analyzed by 5% denaturing (8 M urea) PAGE. SOCS3 and GAPDH mRNA expression was also analyzed by RT-PCR (25). One microgram of RNA was used to reverse transcribe mRNA into cDNA using Moloney murine leukemia virus and GAPDH reverse transcriptase and subjected to PCR with primers specific for mouse SOCS3 and GAPDH for 30 cycles of amplification. Primer sequences were as follows: SOCS3 (sense), 5′-GTTGAGCGTCAAGACCCAGT-3; SOCS3 (antisense), 5′-CACGTTGGAGGAGAGAGGTG-3′; GAPDH (sense), 5′-AACTTTGGCATTGTGGAAGG-3′; and GAPDH (antisense), 5′-CCCTGTTGCTGTAGCCGTAT-3′.

Immunoblotting

Protein (20 μg) in cell lysates were separated by electrophoresis on 8% SDS-polyacrylamide gels and probed with phospho-STAT3 Abs as described previously (25). Membranes were stripped and reprobed for total STAT3 and actin.

Flow cytometry

As described previously (4), cells were stimulated for 5 h with PMA (50 ng/ml) and ionomycin (750 ng/ml), with monensin added for the last 3 h of culture. After surface staining with CD4 Ab, the cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Pharmingen). Staining was performed for IL-17, IFN-γ, IL-10, or IL-2 using PE-or allophycocyanin-conjugated Abs (BD PharMingen), and the cells were quantitated using a FACStar flow cytometer (BD Biosciences). A FITC-, allophycocyanin-, or PE-labeled mAb of the same isotype but irrelevant specificity was used as a negative control in all experiments.

Analysis of RORγt mRNA expression

Expression of RORγt on T cells was detected using real-time PCR with the primers 5′-CCGCTGAGAGGGCTTCAC-3′, and 5′-TGCAGGAGTAGGC CACATTACA-3′ at a final concentration of 800 nM and the FAM-labeled internal probe 5′-AAGGGCTTCTTCCGCCGCAGCCAGCAG-TAMRA-3′ at a final concentration of 150 nM. As an endogenous reference, β_2-microglobulin was simultaneously measured using the primers 5′-CCTGCAGAGTTAAGCATGCCAG-3′ and 5′-TGCTTGATCACAT GTCTCGATCC-3′ (final concentration, 30 nM) and the Texas Red-labeled internal probe 5′-TGGCCGAGCCCAAGACCGTCTAC-3′ (final concentration, 50 nM). All primers and probes were obtained from Integrated DNA Technologies. Multiplex reactions were performed using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and amplified with the cycling parameters 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 s and 60°C for 1 min on a Bio-Rad iCycler.

RNA interference

RNA interference of SOCS3 was done as previously described (26). Dharma*FECT* 1 small interfering siRNA (siRNA) transfection reagent, SMARTpool siRNAs specific for murine SOCS3, and *CONTROL* nontargeting siRNA were purchased from Dharmacon. Naive $CD4^+$ T cells (1×10^6 cells/well in 12-well plates) were transfected with 100 nM control or SOCS3 siRNAs using the Dharma*FECT* 1 reagent and a mouse T cell Nucleofector kit (Amaxa). Twenty-four hours after transfection, T cells were stimulated with IL-6, TGF-β, or both. RORγt expression was measured 48 h later by real-time PCR. IL-17 and Foxp3 were determined 5 days later by flow cytometry.

Transient transfection of SOCS3 promoter-reporter and luciferase assays

A 1619-bp (from −1492 to +127 bp) murine SOCS3 promoter-reporter was used as described previously (26). $CD4^+$ T cells were cultured with anti-CD3/CD28, and 48 h later the cells were washed and seeded in 12-well plates (1.5×10^6 cells/well) and transfected with the murine SOCS3 promoter using the mouse T Cell Nucleofector kit. Transfected cells were treated with medium, IL-6, TGF-β1, or IL-6 plus TGF-β1 for 12 h, and the luciferase activity of each sample was normalized to the total protein concentration in each well. Luciferase activity from the untreated sample was arbitrarily set at 1 for the calculation of fold induction.

Statistical analysis

All experiments were repeated a minimum of three times. For comparisons between samples, levels of significance were determined by Student's *t* test distribution. Values of $p \leq 0.05$ were considered to be statistically significant.

Results

TGF-β inhibits IL-6-induced SOCS3 expression and enhances STAT3 activation

Th1 and Th2 cytokines, IL-2, IL-4, and IFN-γ negatively regulate Th17 cell differentiation (2, 3, 17). TGF-β down-regulates T-bet and GATA3 function and inhibits Th1 and Th2 differentiation and the production of IFN-γ and IL-4. TGF-β can also inhibit IL-2 production (27). Thus, the effects of TGF-β on promotion of Th17 cell differentiation may be secondary to the inhibition of cytokines that block Th17 cell development. We first determined whether this was the case. $CD4^+$ T cells from B6 mice were cultured with anti-CD3 and anti-CD28 alone or with the addition of anti-IL-2, anti-IFN-γ, and anti-IL-4 mAbs in the absence or presence of IL-6 or both IL-6 and TGF-β. Seven days later, IL-17 production by T cells was determined by flow cytometry after restimulation with PMA/ionomycin. The addition of anti-IL-2, anti-IFN-γ, and anti-IL-4 inhibited the production of those cytokines almost completely but did not result in much IL-17 expression. The addition of IL-6 induced small amounts of Th17 cells. The addition of both IL-6 and TGF-β induced substantial Th17 cells (Fig. 1*A*), indicating that

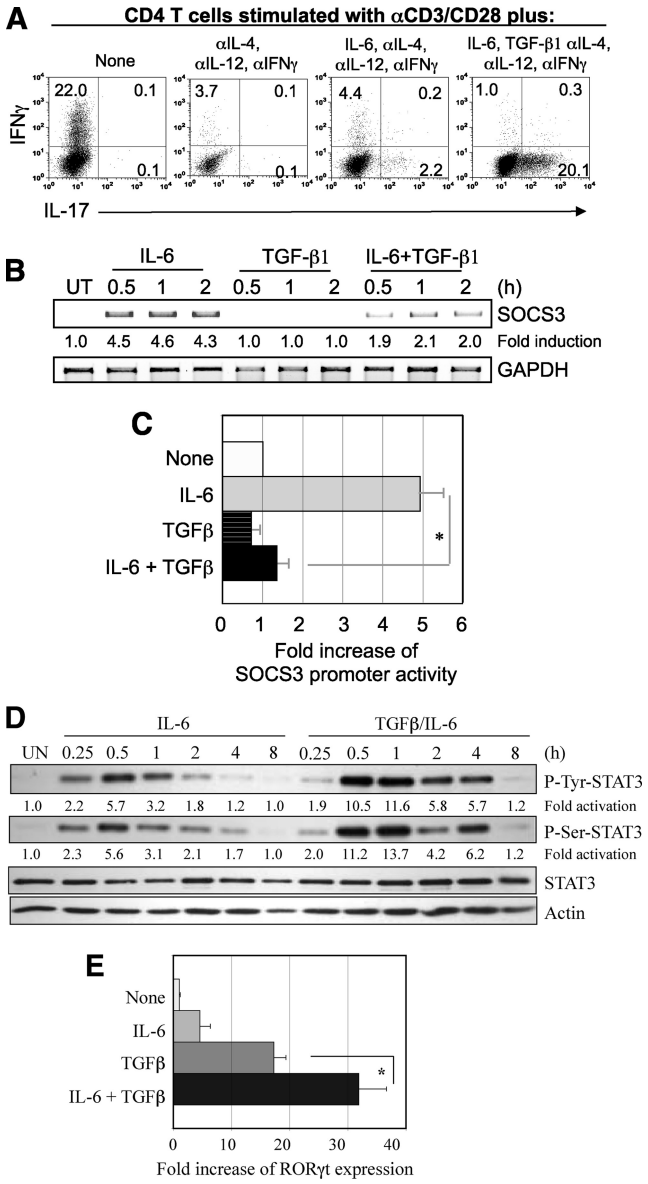

FIGURE 1. Effect of IL-6 and TGF-β1 on Th17 cell development, expression of SOCS3 and RORγt, and activation of STAT3 in CD4$^+$ T Cells. *A*, B6 CD4$^+$ T cells were cultured with anti-CD3/CD28 (αCD3/CD28) in the absence or presence of IL-6 (20 ng/ml), anti-IFN-γ (αIFNγ; 10 μg/ml), anti-IL-12 (αIL-12; 10 μg/ml), anti-IL-4 (αIL-4; 10 μg/ml), and TGF-β (TGF-β1; 5 ng/ml). Seven days later, the cells were restimulated with PMA/ionomycin for 5 h and intracellular IL-17 and IFN-γ production was measured by flow cytometry. *B*, To measure IL-6- and TGF-β (TGF-β1) induction of SOCS3, CD4$^+$ T cells were treated with medium, IL-6 (20 ng/ml), or IL-6 plus TGF-β (IL-6+TGF-β1; 5 ng/ml) for up to 2 h, and total RNA was analyzed by RT-PCR to measure SOCS3 and GAPDH mRNA levels. mRNA levels in the untreated sample (UT) were set at 1.0, and the results are expressed as fold induction over these control levels. *C*, To determine the effect of TGF-β on SOCS3 promoter activity, CD4$^+$ T cells were transfected with a 1619-bp (from -1492 to $+127$ bp) murine SOCS3 promoter and treated with IL-6 (20 ng/ml), TGF-β (5 ng/ml), or both for 12 h, and the luciferase activity of each sample was normalized to the total protein concentration in each well. Luciferase activity from the untreated sample was arbitrarily set at 1 for the calculation of fold induction. *, $p \leq 0.05$. *D*, To evaluate STAT3 activation, CD4$^+$ T cells were incubated with IL-6 (20 ng/ml), or IL-6 plus TGF-β (IL-6 + TGFβ; 5 ng/ml) for up to 8 h, and then cell lysates were immunoblotted with anti-phospho-Tyr-STAT3 (P-Tyr-STAT3) or anti-phospho-Ser-STAT3 (P-Ser-STAT3). The membranes were stripped and reprobed with anti-STAT3 and anti-actin as a loading control. The data shown are representative of at least three experiments. STAT3 phosphorylation level in the untreated sample (UN) was set at 1.0, and results are calculated as fold activation over these control levels. *E*, RORγt expression of B6 CD4$^+$CD25$^-$ T cells cultured with anti-CD3/CD28, anti-IFN-γ, and anti-IL-4 in the absence or presence of IL-6 (20 ng/ml) and TGF-β (5 ng/ml) was determined by real-time PCR 48 h after culture. The data were normalized to a β_2-microglobulin reference. The experiments were repeated three times with consistent results. *, $p \leq 0.05$.

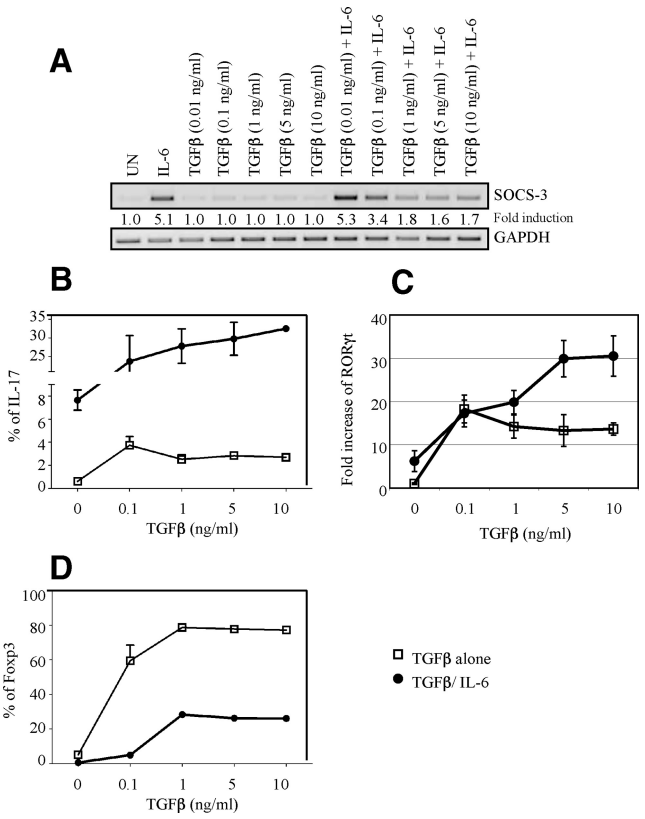

FIGURE 2. Dose response of TGF-β on the expression of SOCS3, RORγt, Foxp3, and Th17 cell development in naive CD4$^+$ T Cells. *A*, B6 CD4$^+$CD25$^-$ T cells were cultured with increasing doses of TGF-β (TGFβ; 0.01 to 10 ng/ml) in the absence or presence of IL-6 (20 ng/ml) for 2 h, and total RNA was analyzed by RT-PCR to measure SOCS3 and GAPDH mRNA levels. mRNA levels in the untreated sample (UN) were set at 1.0, and results are expressed as fold induction over these control levels. *B*, B6 CD4$^+$CD25$^-$ T cells were cultured with anti-CD3/CD28 and anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml), and increasing doses of TGF-β in the presence or absence of IL-6 (20 ng/ml). To measure IL-17 production, 5 days after culture the cells were restimulated with PMA/ionomycin for 5 h and intracellular IL-17 was measured by flow cytometry. *C*, RORγt expression was measured by real-time PCR 48 h after culture. The data were normalized to β_2-microglobulin as a reference. The experiments were repeated three times with consistent results. *D*, Foxp3 expression was determined by flow cytometry 5 days after culture. Representative of three experiments.

IL-6 alone induced weak Th17 cell differentiation, TGF-β was required for substantial Th17 cell development, and the blockade of Th1 and Th2 cytokines did not compensate the function of TGF-β in the induction of Th17 cell differentiation.

IL-6 and IL-21 induce STAT3 activation that in turn regulates the transcription factors RORγt and RORα in the presence of TGF-β and, thus, Th17 cell differentiation (9, 11). Interestingly, although IL-6 or IL-21 can each activate STAT3 and induce a low level of RORγt expression (20), neither is sufficient for Th17 cell

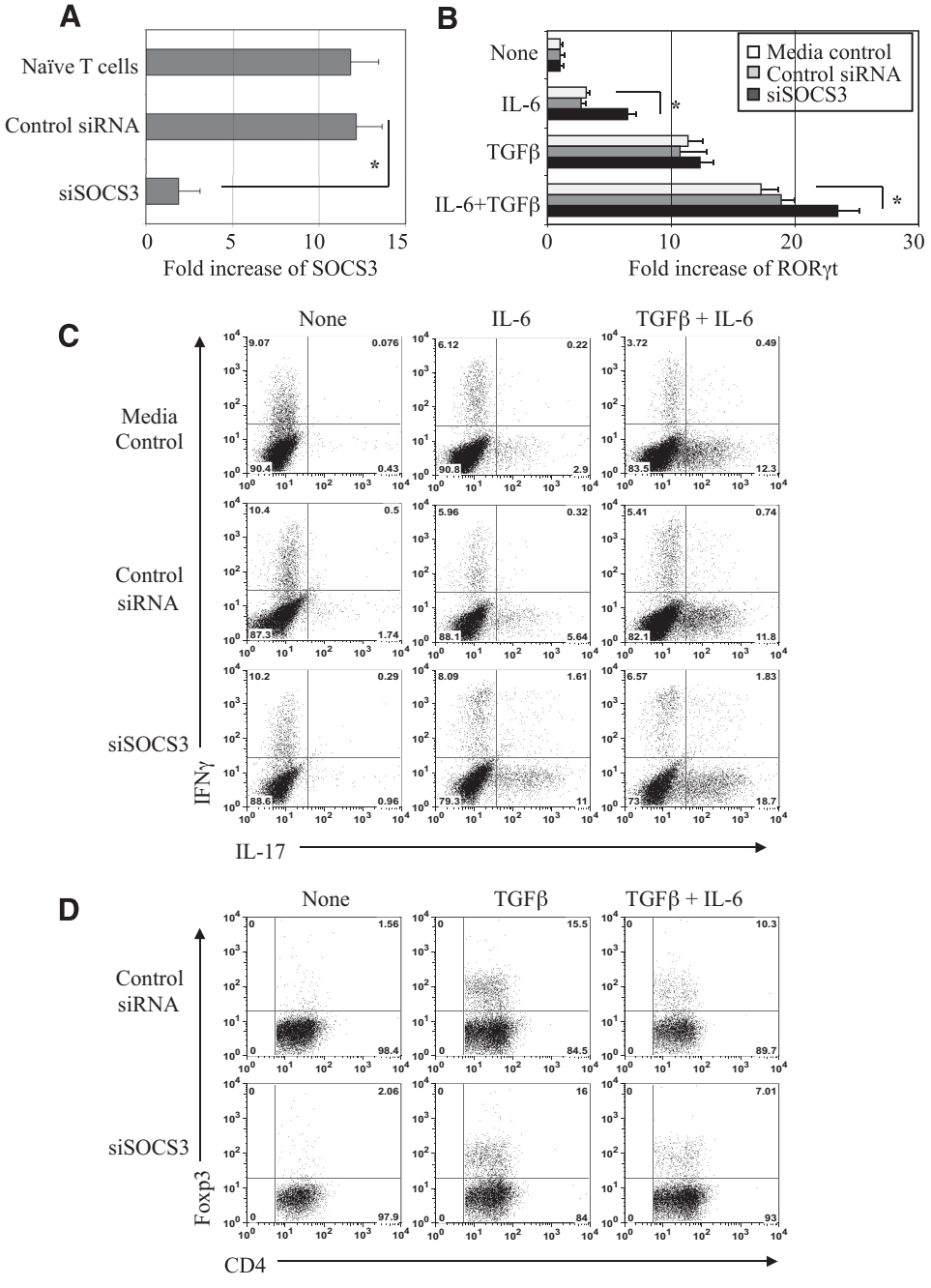

FIGURE 3. Effect of SOCS3 knockdown on RORγt and Th17 cell development. Naive $CD4^+CD25^-$ T cells were transfected with 100 nM control (Control siRNA) or SOCS3 (siSOCS3) siRNAs using the Dharma*FECT* 1 reagent and the mouse T cell Nucleofector kit (Amaxa). Twenty-four hours after transfection, T cells were stimulated with anti-CD3/CD28 mAbs in the presence of IL-6 (20 ng/ml), TGF-β (TGFβ; 5 ng/ml), or both. *A*, SOCS3 expression was measured after 1 h of IL-6 (20 ng/ml) stimulation by real-time PCR. The data were normalized to a β_2-microglobulin reference. *, $p \leq 0.05$. *B*, RORγt expression was measured 48 h later by real-time PCR. The data were normalized to β_2-microglobulin as a reference. *, $p \leq 0.05$. *C*, IL-17 and IFN-γ production was determined 5 days later by flow cytometry. *D*, Foxp3 expression was determined 5 days later by flow cytometry. Data are representative of three experiments.

differentiation without the action of TGF-β. One explanation is that the level of activated STAT-3 by IL-6 or IL-21 is not high enough to reach the threshold to activate the Th17 cell differentiation program and that the action of TGF-β is to enhance STAT3 activation to reach that threshold. IL-6 stimulates STAT3 activation as well as SOCS3 expression in multiple types of cells, including T cells. SOCS3 inhibits STAT3 activation and thus represents a classic feedback inhibitor of cytokine signaling (21, 22). It has been shown that SOCS3 negatively regulates the Th17 cell pathway by the inhibition of STAT3, in that mice with SOCS3 deficiency in hematopoietic and endothelial cells develop severe joint inflammation due to enhanced responsiveness to IL-6 with a high level of production of IL-17, a hallmark cytokine of Th17 cells (28). In mice with SOCS3-deficient T cells, IL-6- and IL-23-dependent STAT3 phosphorylation is enhanced as well as prolonged, and such enhanced signaling is associated with an increase in Th17 cell development both in vitro and in vivo (23). Overexpression of SOCS3 in T cells results in reduced STAT3 phosphorylation in response to IL-6 stimulation, less Th17 cell development, and significantly delayed EAE onset (24). We postulated that TGF-β might inhibit SOCS3 expression induced by IL-6 and IL-21, thus enhancing STAT3 activation and Th17 cell development.

B6 $CD4^+$ T cells were cultured with anti-CD3 and anti-CD28 in the presence of IL-6 alone, TGF-β alone, or IL-6 plus TGF-β. As shown in Fig. 1*B*, IL-6 stimulated naive $CD4^+$ T cell SOCS3 expression at each time point from 0.5 to 2 h, whereas TGF-β alone did not induce SOCS3 expression. Interestingly, treatment with TGF-β inhibited IL-6-induced SOCS3 expression. To determine how TGF-β inhibited IL-6-induced SOCS3 expression, we performed an analysis of potential transcription factor binding sites with the Genomatix MatInspector system. A Smad binding site (from −177 to −185) was identified within the SOCS3 promoter. To determine the effect of TGF-β on SOCS3 promoter activity,

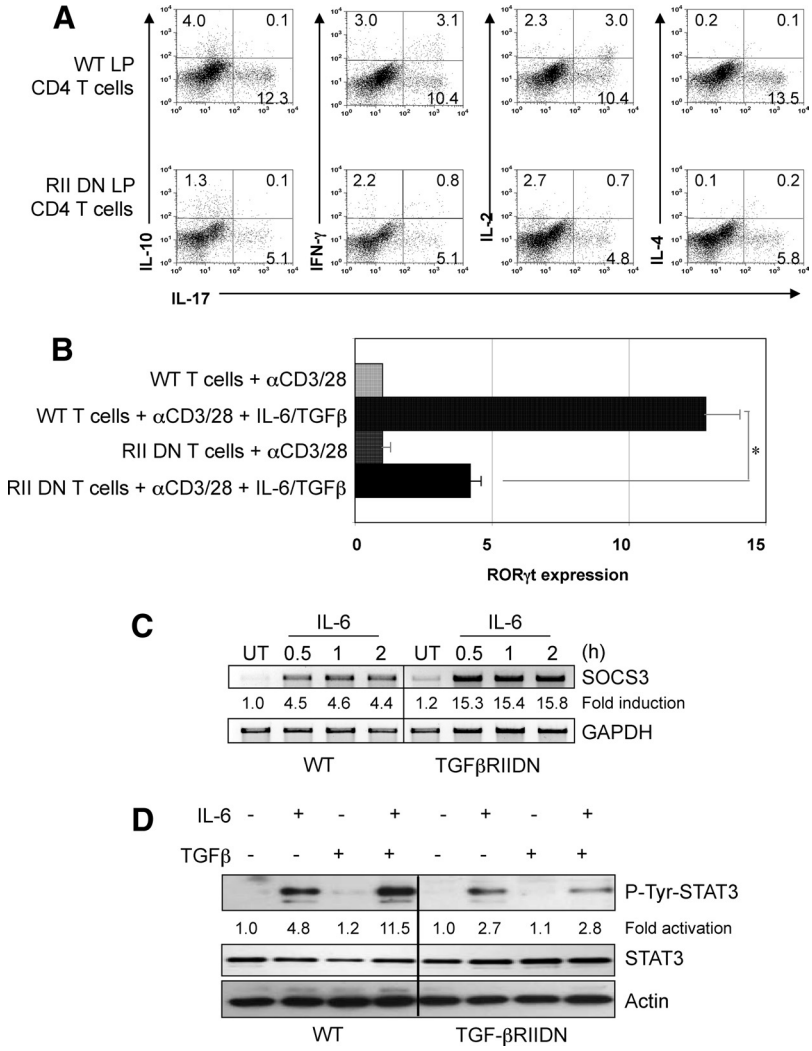

FIGURE 4. Fewer Th17 cells in lamina propria of TGF-βRII DN mice and lower RORγt expression of TGF-βRII DN CD4$^+$ T cells stimulated with IL-6 and TGF-β. *A*, Lamina propria (LP) CD4$^+$ T cells were isolated from WT or TGF-βRII DN B6 mice and stimulated with PMA/ionomycin for 5 h. Intracellular IL-17 and IFN-γ production was measured by flow cytometry. *B*, Splenic CD4$^+$ T cells were isolated from WT or TGF-βRII DN B6 mice and cultured with anti-CD3/CD28 (αCD3/28) in the absence or presence of IL-6 (20 ng/ml), anti-IFN-γ (10 mg/ml), anti-IL-4 (10 mg/ml), and TGF-β (5 ng/ml). RNA was isolated and RORγt expression was analyzed using real-time PCR. Expression of β_2-microglobulin was used as housekeeping gene control. Data are representative of three experiments. *, $p \leq 0.05$. *C*, WT or TGF-βRIIDN CD4$^+$ T cells were treated with medium alone or IL-6 (20 ng/ml) for up to 2 h, and total RNA was analyzed by RT-PCR to measure SOCS3 and GAPDH mRNA levels. mRNA levels in the untreated sample (UT) were set at 1.0, and results were expressed as fold induction over these control levels. *D*, To evaluate STAT3 activation, WT or TGF-βRIIDN CD4$^+$ T cells were incubated with IL-6 (20 ng/ml), TGF-β (5 ng/ml), or IL-6 plus TGF-β for 1 h, and then cell lysates were immunoblotted with anti-phospho-Tyr-STAT3 (P-Tyr-STAT3). The membranes were stripped and reprobed with anti-STAT3 and anti-actin as a loading control. The STAT3 phosphorylation level in the untreated sample was set at 1.0, and results are calculated as fold activation over these control levels. The data shown are representative of at least three experiments.

CD4$^+$ T cells were transfected with a 1619-bp (from -1492 to $+127$ bp) murine SOCS3 promoter-reporter as previously described (26) and treated with IL-6, TGF-β, or both IL-6 and TGF-β. As shown in Fig. 1*C*, TGF-β inhibited IL-6-induced SOCS3 promoter activity. To determine whether TGF-β inhibition of IL-6-induced SOCS3 expression would result in enhanced STAT3 activation, STAT3 phosphorylation at both Tyr and Ser sites was measured at a series of time points from 15 min to 8 h. IL-6 stimulated STAT3 activation in naive CD4$^+$ T cells by 15 min, which peaked at 1 h and then declined at 2 h. Treatment with TGF-β did not affect IL-6-induced STAT3 activation at the earlier time point (15 min), but enhanced STAT-3 activation at 0.5 h and at each subsequent time point (Fig. 1*D*). Notably, TGF-β not only enhanced but also prolonged IL-6-induced STAT-3 activation, in that IL-6-stimulated-STAT3 activation waned at 2 h and almost disappeared at 4 h. In contrast, STAT3 activation was still strong at 4 h when naive CD4$^+$ T cells were treated with both IL-6 and TGF-β. TGF-β has been shown to promote regulatory T cell (Treg) expansion. To determine whether the effect of TGF-β on STAT3 activation could be due to reduced numbers of effector T cells, CD4$^+$CD25$^-$ T cells were cultured with anti-CD3 and anti-CD28 in the presence of IL-6, TGF-β, or IL-6 plus TGF-β, and SOCS3 expression and STAT3 activation were determined at different time points from 0.5 to 4 h. IL-6 induced both SOCS3 expression and STAT3 activation, whereas TGF-β inhibited SOCS3 expression and enhanced STAT3 activation (data not shown), similarly as for unseparated CD4$^+$ T cells. We then examined whether this TGF-β-stimulated, prolonged STAT3 activation could enhance RORγt expression of naive CD4$^+$ T cells. TGF-β induced RORγt expression in naive CD4$^+$CD25$^-$ T cells, which was enhanced when cultured with both IL-6 and TGF-β. IL-6 alone induced little RORγt (Fig. 1*E*).

It has been shown recently that TGF-β plays an important role in both Foxp3 and RORγt induction, whereas IL-6/STAT3 suppresses Foxp3 induction and enhances RORγt expression (18, 19). To further determine the role played by TGF-β inhibition of IL-6-induced SOCS3 on Foxp3 and RORγt expression in naive CD4$^+$ T cells, CD4$^+$CD25$^-$ T cells were stimulated with increasing doses of TGF-β in the absence or presence of IL-6, and SOCS3, Foxp3 and RORγt expression and IL-17 production were determined. As shown in Fig. 2*A*, TGF-β alone did not induce SOCS3 expression at all doses; however, TGF-β inhibited IL-6-induced SOCS3 expression in a dose-dependent manner. TGF-β did not affect IL-6-induced SOCS3 expression at 0.01 ng/ml, modestly inhibited SOCS3 at 0.1 ng/ml, and then enhanced inhibition of SOCS3 with increasing concentration of TGF-β (1–10 ng/ml). TGF-β alone induced low levels of IL-17 production and RORγt expression and high levels of Foxp3 expression (Fig. 2, *B–D*). Interestingly, TGF-β induction of IL-17 production and RORγt expression was inversely correlated with the TGF-β dose, in that TGF-β induced more IL-17 and RORγt at low doses (0.1 ng/ml) and IL-17 and RORγt expression decreases at higher doses of

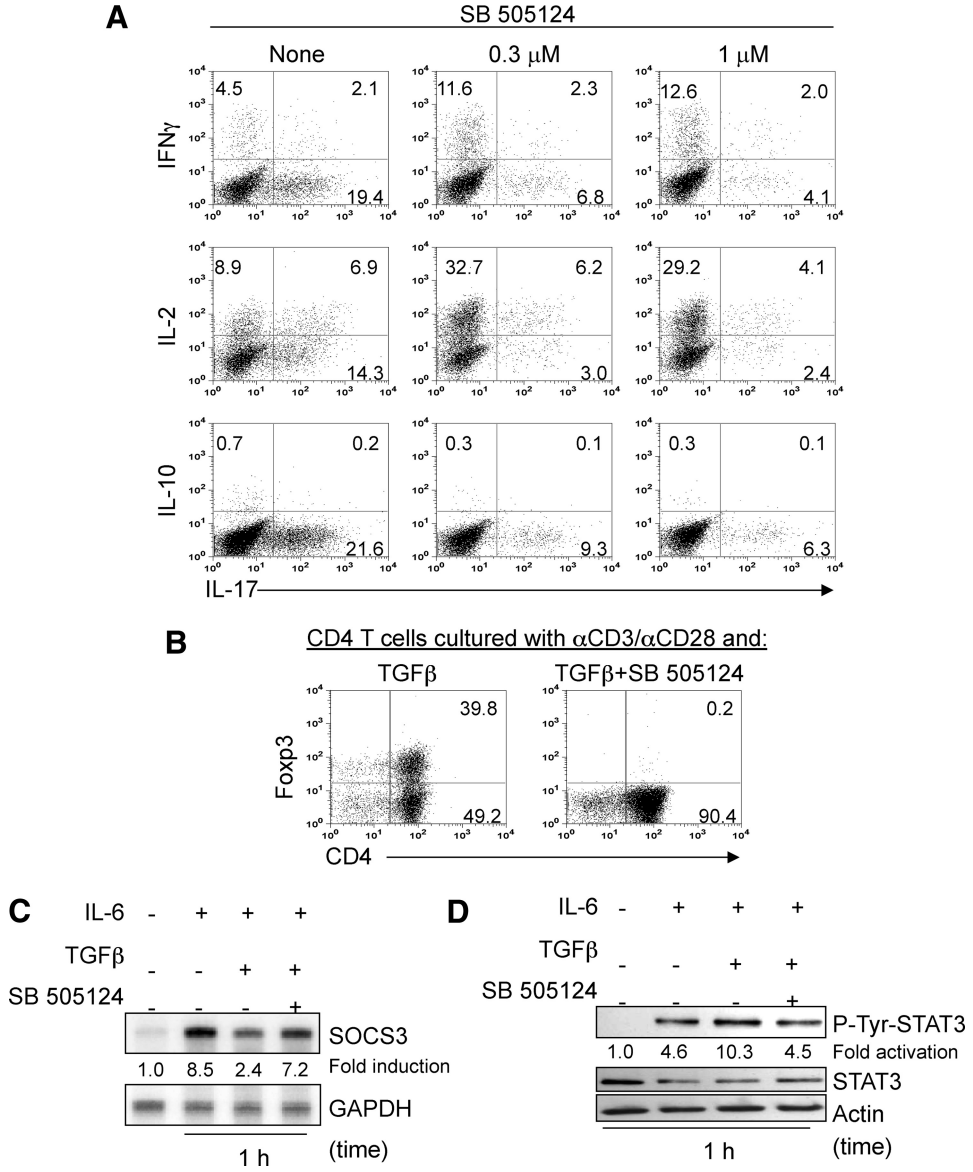

FIGURE 5. The effect of the TGF-βRI kinase inhibitor SB505124 on Th17 and Treg differentiation, STAT3 activation, and SOCS3 mRNA expression in $CD4^+$ T cells. *A*, As described in the legend to Fig. 1, B6 $CD4^+$ T cells were cultured with anti-CD3/CD28 in the absence or presence of IL-6 (20 ng/ml), anti-IFN-γ (10 mg/ml), anti-IL-4 (10 mg/ml), and TGF-β (5 ng/ml) in the absence or presence of different doses of SB505124 (0–1 μM). Seven days later, the cells were restimulated with PMA/ionomycin for 5 h and intracellular IL-17, IL-2, IL-10, and IFN-γ production was measured by flow cytometry. *B*, B6 $CD4^+$ T cells were cultured with anti-CD3/CD28 (αCD3/αCD28) and TGF-β (5 ng/ml) in the absence or presence 1 μM SB505124. Seven days later, Foxp3 expression was determined by flow cytometry. *C*, $CD4^+$ T cells were treated with IL-6 (10 ng/ml), IL-6 plus TGF-β (5 ng/ml), or IL-6 plus TGF-β and SB505124 (0–1 μM) for 1 h. Total cellular mRNA was analyzed for SOCS3 and GAPDH mRNA expression by RPA. Basal levels in the untreated sample were set at 1.0, and results are expressed as fold induction over these control levels. *D*, To evaluate the effect of the Smad inhibitor SB505124 on STAT3 activation, $CD4^+$ T cells were incubated with IL-6 (10 ng/ml), IL-6 plus TGF-β (5 ng/ml), or IL-6 plus TGF-β and 1 μM SB505124 for 1 h and then cell lysates were immunoblotted with anti-phospho-Tyr-STAT3 (P-Tyr-STAT3). The membranes were stripped and reprobed with anti-STAT3 and anti-actin as a loading control. The data shown are representative of at least three experiments.

TGF-β. However, TGF-β induction of Foxp3 was dose dependent, in that Foxp3 expression increased with the increasing concentration of TGF-β. In contrast, in the presence of IL-6, TGF-β induced IL-17 production and RORγt expression in a dose-dependent manner that correlated with the TGF-β dose-dependent inhibitory effect on SOCS3 expression. IL-6 inhibited Foxp3 induction by TGF-β at all doses. These data indicate that TGF-β inhibition of SOCS3 expression contributes to the effects of IL-6 on IL-17 production and RORγt expression but has no direct effect on TGF-β induction of Foxp3 expression. Collectively, these results demonstrate that TGF-β inhibits IL-6-induced SOCS3 expression and thus promotes STAT3 activation, RORγt expression, and Th17 cell differentiation of naive T cells.

SOCS3 knockdown in naive $CD4^+CD25^-$ T cells partially compensates for the action of TGF-β on RORγt expression and Th17 cell development

To determine the role played by TGF-β inhibition of IL-6-induced SOCS3 expression on Th17 cell development, SOCS3 expression in $CD4^+CD25^-$ T cells was knocked down by siRNAs specific for murine SOCS3. Nontargeting siRNA was used as control. Transfection of $CD4^+CD25^-$ T cells with SOCS3

siRNA, but not control siRNA, greatly inhibited IL-6-induced SOCS3 expression (Fig. 3*A*). Transfected $CD4^+CD25^-$ T cells were cultured with anti-CD3/CD28 mAbs in the presence of IL-6, TGF-β, or IL-6 and TGF-β. SOCS3 knockdown enhanced RORγt expression in $CD4^+CD25^-$ T cells (Fig. 3*B*) and Th17 development (Fig. 3*C*) in response to IL-6. SOCS3 knockdown also enhanced RORγt expression and Th17 development if both IL-6 and TGF-β were present, suggesting that TGF-β inhibition of SOCS3 expression partially contributes to RORγt expression and Th17 development. However, TGF-β was still required for maximal Th17 cell development, even in the absence of SOCS3.

To determine the role of TGF-β inhibition of SOCS3 on Foxp3 and RORγt expression induced by TGF-β, SOCS3 expression in $CD4^+CD25^-$ T cells was knocked down by siRNA specific for murine SOCS3 as described above. Nontargeting siRNA was used as control. As shown previously, TGF-β alone induced both RORγt and Foxp3 (Fig. 3, *B* and *D*). IL-6 inhibited TGF-β induction of Foxp3 (Fig. 3*D*). SOCS3 knockdown did not affect the induction of RORγt and Foxp3 by TGF-β alone but enhanced IL-6 inhibition of Foxp3 expression induced by TGF-β (Fig. 3, *B* and *D*). Collectively, these data indicate that TGF-β inhibition of SOCS3 mainly enhances the function of the IL-6/STAT3 pathway on the inhibition of Foxp3 expression.

Higher expression of SOCS3 and lower STAT3 activation in TGF-βRII DN T cells compared with WT T cells

To further examine the possibility that TGF-β inhibits SOCS3 expression and thus enhances STAT3 activation, we determined SOCS3 expression and STAT3 activation in $CD4^+$ T cells from TGF-βRII DN transgenic mice, whose TGF-β signaling is greatly impaired (29). Because the intestinal lamina propria is the only site identified to date as having a population of naturally occurring Th17 cells (8), we first compared Th17 cell development in the intestinal lamina propria of WT and TGF-βRII DN mice. Th17 cells were reduced in the intestinal lamina propria in TGF-βRII DN mice compared with that of WT mice (Fig. 4*A*). To determine the effect of TGF-β on IL-6-induced SOCS3 expression and STAT3 activation, $CD4^+$ T cells from WT and TGF-βRII DN mice were stimulated with IL-6 and TGF-β alone or together, and SOCS3 expression and STAT3 activation were assessed. As shown in Fig. 4, *C* and *D*, IL-6 induced a higher level of SOCS3 expression in TGF-βRII DN $CD4^+$ T cells compared with that of WT $CD4^+$ T cells, suggesting that endogenous TGF-β in WT $CD4^+$ T cells could inhibit SOCS3 expression. TGF-β no longer enhanced STAT-3 activation induced by IL-6 in TGF-βRII DN $CD4^+$ T cells (Fig. 4*D*). There was also a lower level of RORγt expression and Th17 cell differentiation in IL-6 plus TGF-β-stimulated $CD4^+$ T cells from TGF-βRII DN mice (Fig. 4*B*).

TGF-βRI kinase inhibitor blocks effect of TGF-β on Th17 cell differentiation

The active form of TGF-β initially engages a receptor comprised of TGF-βRI and TGF-βRII. Binding results in the activation of downstream signal transduction by either a Smad-dependent or a Smad-independent pathway (27). The Smad-dependent pathway involves phosphorylation of Smad2 and Smad3, which translocate into the nucleus in a complex with Smad4. The Smad-independent pathway involves the activation of other mediators such as the MAPKs ERK, Jun N-terminal kinase (JNK), and Rho family members. We next determined which pathway mediates the effect of TGF-β on SOCS3 expression, STAT3 activation, and Th17 cell differentiation. $CD4^+$ T cells were cultured under Th17 polarization conditions with IL-6 and TGF-β in the absence or presence of an inhibitor of TGF-βRI, SB505124. This small molecule inhibitor selectively and concentration dependently inhibits TGF-β-induced Smad activation through ALK-4, ALK-5, and ALK-7 (30). As shown in Fig. 5*A*, the addition of SB505124 greatly inhibited $CD4^+$ T cell IL-17 production (~80%) but increased IFN-γ and IL-2 production. The addition of SB505124 had no effect on IL-10 production. TGF-β not only promotes Th17 cell development but also promotes Treg cells by the induction of Foxp3 (31). When SB505124 was added at the dose of 1 μM to $CD4^+$ T cells cultured with TGF-β, Foxp3 expression induced by TGF-β was completely abrogated (Fig. 5*B*), suggesting that this dose of SB505124 completely blocks the Smad-dependent TGF-β signaling pathway. However, the addition of SB505124 at the same dose in Th17 culture conditions inhibited only ~ 80% of IL-17 production (Fig. 5*A*). These data raise the possibility that TGF-β induction of Foxp3 expression and Treg cell differentiation is Smad dependent, and although TGF-β promotion of Th17 cell development is mediated predominantly by the Smad-dependent pathway, a Smad-independent pathway could also be involved, but to a lesser extent. The addition of SB505124 blocked TGF-β inhibition of IL-6-induced SOCS3 expression (Fig. 5*C*) and resulted in a lower level of STAT3 activation (Fig. 5*D*).

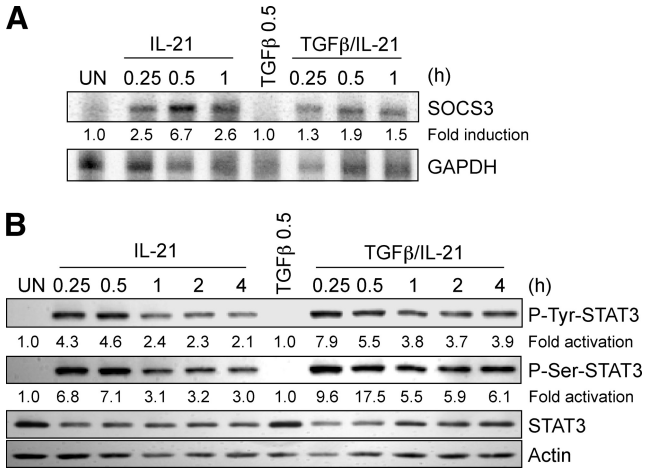

FIGURE 6. IL-21- and TGF-β-induced SOCS3 expression and STAT3 activation in $CD4^+$ T cells. *A*, $CD4^+$ T cells were treated with medium, IL-21 (10 ng/ml), or IL-21 plus TGF-β (TGFβ/IL-21; 5 ng/ml) for up to 1 h, and total RNA was analyzed by RPA to measure SOCS3, and GAPDH mRNA levels. mRNA levels in the untreated sample (UN) were set at 1.0, and results are expressed as fold induction over these control levels. *B*, To evaluate STAT3 activation, $CD4^+$ T cells were incubated with IL-21 (10 ng/ml), TGF-β (5 ng/ml), or IL-21 plus TGF-β for up to 4 h and then cell lysates were immunoblotted with anti-phospho-Tyr-STAT3 (P-Tyr-STAT3) or anti-phospho-Ser-STAT3 (P-Ser-STAT3). The membranes were stripped and reprobed with anti-STAT3 and anti-actin as loading controls. The STAT3 phosphorylation level in the untreated sample was set at 1.0, and results were calculated as fold activation over these control levels. The data shown are representative of at least three experiments.

TGF-β inhibits IL-21-induced SOCS3 expression and enhances STAT3 activation

The combination of IL-21 and TGF-β is able to differentiate naive T cells into Th17 cells in the absence of IL-6 (10–12). Although IL-21 has been shown to activate STAT3, a key signaling event for Th17 cell differentiation, it is still unclear whether it also induces SOCS3 as does IL-6. B6 $CD4^+$ T cells were cultured with anti-CD3 and anti-CD28 plus IL-21 in the absence or presence of

TGF-β, and SOCS3 expression and STAT-3 activation were measured. Similarly as $CD4^+$ T cells stimulated with IL-6, IL-21 also stimulated SOCS3 expression, and treatment with TGF-β greatly inhibited such SOCS3 expression (Fig. 6*A*). Interestingly, similar to IL-6, IL-21-stimulated STAT3 activation started at 15 min, peaked at 0.5–1 h, and then subsided gradually (Fig. 6*B*). Treatment with TGF-β not only enhanced but also prolonged IL-21-induced STAT3 activation. This finding suggests that TGF-β inhibition of SOCS3 and the promotion of STAT3 activation is not specific for IL-6 signaling but presents a common pathway for TGF-β modulation of Th17 cell differentiation.

Discussion

Our data demonstrate that TGF-β promotes as well as prolongs STAT-3 activation induced by IL-6 and IL-21 through the inhibition of SOCS3 expression. The inhibition of SOCS3 by TGF-β releases the negative regulation of STAT3 activation by SOCS3 and results in enhanced STAT3 activation and Th17 cell differentiation. This crosstalk between TGF-β and the downstream signaling molecules of IL-6 and IL-21 plays a crucial role for Th17 cell development and could be at least one of the mechanisms by which TGF-β affects Th17 cell differentiation. A recent report by Yoshimura's group showed that Th17 cell differentiation was impaired in SOCS1-deficient mice, probably due to STAT3 suppression by enhanced SOCS3 expression through hyper-STAT1 activation (24). Interestingly, TGF-β-mediated Smad transcriptional activity was severely impaired in these SOCS1-deficient T cells, and in this case TGF-β signaling no longer inhibited SOCS3 expression and thus resulted in a high level of SOCS3 expression and decreased STAT3 activation and defective Th17 cell development. IFN-γ and IL-4 have been shown to inhibit the Th17 pathway; however, the mechanisms involved are still unknown. In contrast to TGF-β, which inhibits SOCS3 expression, IFN-γ and IL-4 are able to induce SOCS3 expression (32). However, IL-4-induced STAT6 activation was not affected in SOCS3-deficient T cells, indicating that it is not likely that SOCS3 directly regulates IL-4 signaling. Instead, IFN-γ and IL-4 induction of SOCS3 expression could contribute to their inhibitory effect on Th17 cell differentiation.

It has been shown recently that TGF-β plays an important role in both Foxp3 and RORγt induction, whereas IL-6/STAT3 suppresses Foxp3 induction (18, 19). Interestingly, knockdown of SOCS3 expression in $CD4^+CD25^-$ T cells by siRNA did not affect TGF-β induction of Foxp3 and RORγt but enhanced the ability of IL-6 to induce RORγt and Th17 development while suppressing Foxp3, suggesting that TGF-β inhibition of SOCS3 mainly enhances the IL-6/STAT3 pathway. SOCS3 knockdown can partially compensate for the role of TGF-β; however, TGF-β is still required for maximum Th17 cell development even in the absence of SOCS3.

Both Smad-dependent and Smad-independent pathways have been implicated in the function of TGF-β (27). It is still unclear which pathway is used for Th17 cell development. When $CD4^+$ T cells were cultured in the Th17 polarization condition with IL-6 and TGF-β in the presence of SB505124, the inhibitor of TGF-βRI kinase that specifically inhibits the Smad-dependent pathway, the TGF-β-driven Th17 cell pathway was greatly blocked in that addition of SB505124 blocked TGF-β-induced inhibition of SOCS3 expression and the enhancement of STAT3 activation, as well as Th17 cell differentiation. Of note, when SB505124 was added at the dose of 1 μM into $CD4^+$ T cells cultured with TGF-β, Foxp3 expression induced by TGF-β was completely abrogated, suggesting that SB505124 at the dose of 1 μM could completely block the Smad-dependent TGF-β signaling pathway. However, the addition of SB505124 at the same dose in Th17 culture condition inhibited ~80% of IL-17 production. These data raise the possibility that TGF-β induction of Foxp3 expression and Treg cell differentiation is Smad dependent and although TGF-β promotion of Th17 cell development is mediated predominantly by the Smad-dependent pathway, a Smad-independent pathway could also be involved, but to a lesser extent. The SB505124 inhibition of Th17 cell development from our study suggests that this TGF-β inhibitor could work as well on various autoimmune diseases that are mediated by Th17 cells.

Disclosures

The authors have no financial conflict of interest.

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