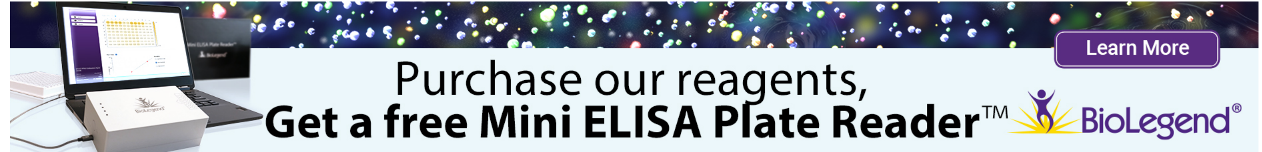

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CUTTING EDGE

Cutting Edge: Foxp3$^+$CD4$^+$CD25$^+$ Regulatory T Cells Induced by IL-2 and TGF-β Are Resistant to Th17 Conversion by IL-6[1]

Song Guo Zheng,[2] Juhua Wang, and David A. Horwitz[2]

***TGF-β has pleiotropic effects on T cell differentiation that are determined by other cytokines in the local environment. Whereas IL-2 and TGF-β induce naive T cells to become forkhead/winged helix transcription factor (Foxp3) positive regulatory cells (iTregs), the combination of IL-6 and TGF-β induces IL-17-producing cells (Th17). Moreover, IL-6 can use TGF-β produced by thymus-derived natural regulatory T cells (nTregs) to convert them to Th17 cells. In this study, we report a major difference between iTregs and nTregs. Treatment of iTregs with IL-6 did not affect Foxp3 expression, and their suppressive activity in vitro and in vivo was intact. To explain this difference between nTregs and iTregs, we found that IL-2 and TGF-β down-regulate IL-6 receptor expression and IL-6 signaling. The resistance of iTregs to Th17 conversion suggests that they can function more effectively than nTregs in an inflammatory milieu and emphasizes the central role of IL-2 in combination with TGF-β to maintain immunologic homeostasis. The Journal of Immunology,* 2008, 180: 7112–7116.**

Antigen-activated naive T cells can become either effector cells that protect the host from harmful microbial pathogens or regulatory/suppressor cells that maintain immunologic homeostasis and prevent autoimmunity. It has become evident that cytokines such as TGF-β can promote both effector and regulatory cell differentiation pathways depending upon other cytokines present in the local environment. T cells activated with IL-2 and TGF-β become forkhead/winged helix transcription factor (Foxp3)[3] positive regulatory T cells (1, 2), whereas activation with IL-6 and TGF-β results in Th17 cells (3–6). The control of these opposite differentiation pathways is not well understood and is a central question.

IL-2 and TGF-β have crucial roles in the differentiation and/or function of Foxp3$^+$ regulatory T cells. IL-2 is required for the maintenance of Foxp3 expression (7). Both mouse and human CD4$^+$CD25$^-$ cells activated in the presence of IL-2 and TGF-β express Foxp3 and become induced CD25$^+$ regulatory T cells (iTregs) that produce TGF-β following restimulation (8, 9). IL-2 and TGF-β are also required for the maintenance of Foxp3 by both natural regulatory T cells (nTregs) and iTregs (10). However, the development of nTregs does not depend upon either IL-2 or TGF-β (1, 11). Foxp3$^+$ regulatory T cells (Tregs) can produce soluble TGF-β, and their suppressive activity is dependent upon TGF-β signaling (12, 13).

In this study we have compared the effects of IL-6 on nTregs and iTregs. As reported by others, IL-6 induces nTregs to become Th17 in a TGF-β-dependent manner (14). By contrast, iTregs failed to become Th17 when stimulated with IL-6. Both nTregs and iTregs had the ability to suppress T cell proliferation in vitro and the development of a chronic graft-vs-host disease (GVHD) with a lupus-like syndrome in vivo. However, the inhibitory activity of nTregs, but not iTregs, decreased markedly following the treatment of these cells with IL-6. Rather than attributing these distinctive characteristics to a lineage difference, we found that the activating nTregs with IL-2 and TGF-β down-regulated IL-6 receptor expression and signaling, thus enabling them to also become resistant to Th17 conversion.

Materials and Methods

Mice

Female DBA/2, C57BL/6, and (DBA/2 × C57BL/6) F1 mice were purchased from The Jackson Laboratory and Foxp3^{gfp} mice were a gift from Dr. A. Y. Rudensky (University of Washington, Seattle, WA). All animals were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Southern California Committee for the Use and Care of Animals (Los Angeles, CA).

In vitro cell stimulation and suppressor assay

Splenic or thymic CD4$^+$ and naive CD4$^+$CD25$^-$ cells were isolated by negative and positive selection, respectively (8, 9). CD4$^+$CD25$^+$CD62L$^+$ CD44^{low} cells were obtained from wild-type mice and CD4$^+$CD62L$^+$GFP$^+$

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[3] Abbreviations used in this paper: Foxp3, forkhead/winged helix transcription factor; Treg, regulatory T cell; nTreg, natural CD4$^+$CD25$^+$ Treg; iTreg, CD4$^+$CD25$^+$ Treg generated ex vivo with IL-2 and TGF-β; GVHD, graft-versus-host disease.

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and $CD4^+CD62L^+GFP^-$ cells were obtained from knock-in mice by cell sorting. The respective GFP populations were >99% pure. iTregs induced from naive $CD4^+CD25^-$ cells of wild-type mice were generated using IL-2 and TGF-β1 (R&D Systems) as previously described (1). iTregs from Foxp3 knock-in mice were prepared by stimulating purified $CD4^+GFP^-$ cells with anti-CD3/CD28-coated beads (1:5) and IL-2 plus TGF-β1 and sorting the induced GFP^+ cells. To generate Th17 cells, naive T or $CD4^+$ cells were stimulated with soluble anti-CD3 (0.5 μg/ml) in the presence of gamma-irradiated APC (30 Gy) or immobilized anti-CD3 (1 μg/ml) and 10 μg/ml soluble anti-CD28 in cultures containing TGF-β1 (2 ng/ml), IL-6, anti-IL-4, and anti-IFN-γ (10 μg/ml) (BD Pharmingen). In other experiments, nTregs or iTregs were preactivated with anti-CD3/CD28-coated beads (1:5) and IL-2 with or without TGF-β for 3 days before stimulation with IL-6. Serum-free medium (AIM-V) was used in the cultures. To measure suppression, CFSE-labeled T cells were stimulated with soluble anti-CD3 (0.25 μg/ml) with irradiated APC (1:1). To the responder cells, Tregs and iTregs stimulated with or without IL-6 were added at a 1:4 ratio and suppression of cycling CFSE-labeled T cells was assessed as described previously (8, 9).

Cytokine staining and production

To determine intracellular IL-17, the cells were restimulated with PMA and ionomycin for 5 h and brefeldin A for 4 h. Cells were stained for surface CD4, fixed, permeabilized, and then stained for IL-17 and/or Foxp3 (eBioscience). On resting or activated nTregs and iTregs we determined by flow cytometry membrane-bound mature TGF-β (anti-TGF-β (clone 29C7); eBioscience), IL-6R (anti-CD126 from BD Pharmingen; anti-CD130 from Medical & Biological Laboratories), and anti-phospho-Stat3 (BD Pharmingen). Soluble IL-17 and active TGF-β in the supernatants were determined by an ELISA.

Real-time PCR

Total RNA was extracted with the RNeasy mini kit (Qiagen). cDNA was generated using a Omniscript RT kit (Qiagen). Smad7 mRNA expression was quantified with ABsolute SYBER Green ROX mix (Thermo). The gene-expression results were expressed as arbitrary units relative to the expression of the gene encoding β-actin. Sequences for Smad7 primers were available upon request.

Induction of GVHD and adoptive transfers

A chronic GVHD with a lupus-like syndrome was induced in DBF1 mice as described previously (8) by injecting i.v. 80 $\times$ 10^6 D2 spleen cells. Other groups received this number of D2 cells plus 5 $\times$ 10^6 nTregs or iTregs stimulated with or without IL-6. Before transfer and 12 days thereafter the animals were bled, serum IgG and anti-dsDNA autoantibodies were measured by an ELISA (8), and animal survival was monitored by Kaplan-Meier survival curves and the log rank test using GraphPad4 PRISM software (GraphPad).

Results and Discussion

IL-2 and TGF-β-induced Tregs, iTregs, are resistant to Th17 conversion when stimulated with IL-6

As described by others, naive T cells activated with IL-6 and TGF-β and nTregs stimulated with IL-6 can produce IL-17 (3–6, 14). Although both nTregs and iTregs did not express IL-17 when stimulated with anti-CD3/CD28 without IL-6, we confirmed that exogenous IL-6 was able to induce nTregs to become Th17 cells (14) (Fig. 1*A*). In sharp contrast, iTregs generated ex vivo with IL-2 and TGF-β failed to become Th17 cells when stimulated with IL-6. Because naive T cells require TCR stimulation to become iTregs, we considered the possibility that activation of nTregs might make them resistant to IL-6. However, previously activated nTregs produced even more IL-17 than resting nTregs (results not shown).

We next purified Treg subsets using Foxp3 knock-in mice because $CD4^+CD25^+$ preparations may be contaminated with non-Tregs. In addition, in some experiments we prepared nTregs and generated iTregs from the thymus because splenic $Foxp3^+$ Tregs are mixture of both populations and markers are not available to distinguish one from the other. Fig. 1*B* again shows that IL-6 can induce 20–30% of GFP^+ nTregs to become Th17. Thymic iTregs prepared from GFP^- cells were completely resistant to this effect of IL-6. In addition to intracellular IL-17, IL-6 induced nTregs but not iTregs to secrete IL-17 into culture supernatants (Fig. 1*C*), We also observed that IL-6 decreased Foxp3 expressed by nTregs, but expression of this transcription factor by iTregs was unaffected by this cytokine (Fig. 1*B*).

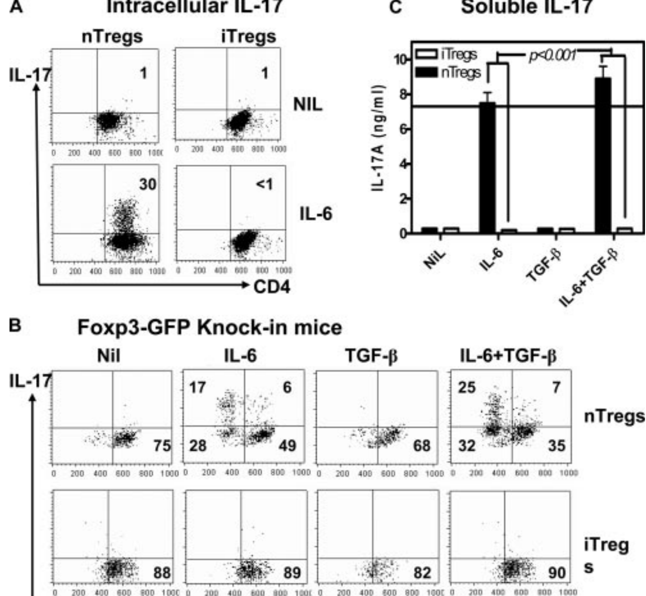

FIGURE 1. iTregs but not nTregs are resistant to Th17 cell conversion when stimulated with IL-6. *A*, iTregs were induced from naive $CD4^+$ cells by stimulation with anti-CD3/28 beads, IL-2, and TGF-β as described in *Materials and Methods*. The iTreg and nTreg ($CD4^+CD25^+CD62L^+$) cells sorted from spleen were then stimulated with immobilized anti-CD3 and soluble anti-CD28 for 3 days with anti-IL-4 and anti-IFN-γ with or without (NIL) IL-6 in the absence of TGF-β1. Results are representative of six independent experiments. *B*, Using the protocol described in *A*, iTregs and nTregs were prepared from thymic single positive $CD4^+$ cells of GFP Foxp3 knock-in mice and the purified GFP^+ subsets were TCR-stimulated with or without IL-6. GFP and IL-17 expressions were determined by flow cytometry simultaneously. Total cell numbers and viability of both Tregs stimulated with or without IL-6 were not significantly different. Data are representative of three separate experiments. *C*, IL-17 levels (mean $\pm$ SEM of three experiments) in supernatants from stimulated Tregs were assayed by ELISA. Horizontal line indicates the level produced by naive $CD4^+$ cells primed with IL-6 and TGF-β.

We considered whether the differences in Th17 conversion between nTregs and iTregs could possibly be explained by quantitative differences in TGF-β production between these two subsets. However, expression of membrane-bound TGF-β and the production of soluble active TGF-β by both subsets were similar (results not shown), Moreover, the addition of a large excess of exogenous TGF-β to a GFP^+ Treg population enhanced IL-17 production by nTregs, but here also iTregs stimulated with IL-6 did not produce IL-17 (Fig. 1*B*).

Decreased suppressive activity of nTregs, but not iTregs, after treatment with IL-6

We also assessed suppressive activities of nTregs and iTregs before and after stimulation with IL-6. The ability of nTregs to suppress the T cell proliferation was also decreased. The experiments shown in Fig. 2, *A* and *B*, reveal that following activation with IL-6, two-thirds of the inhibitory activity of nTregs was lost whereas suppression by iTregs was completely intact (Fig. 2, *A* and *B*). Thus, decreased suppressive activity correlated with loss of Foxp3 expression.

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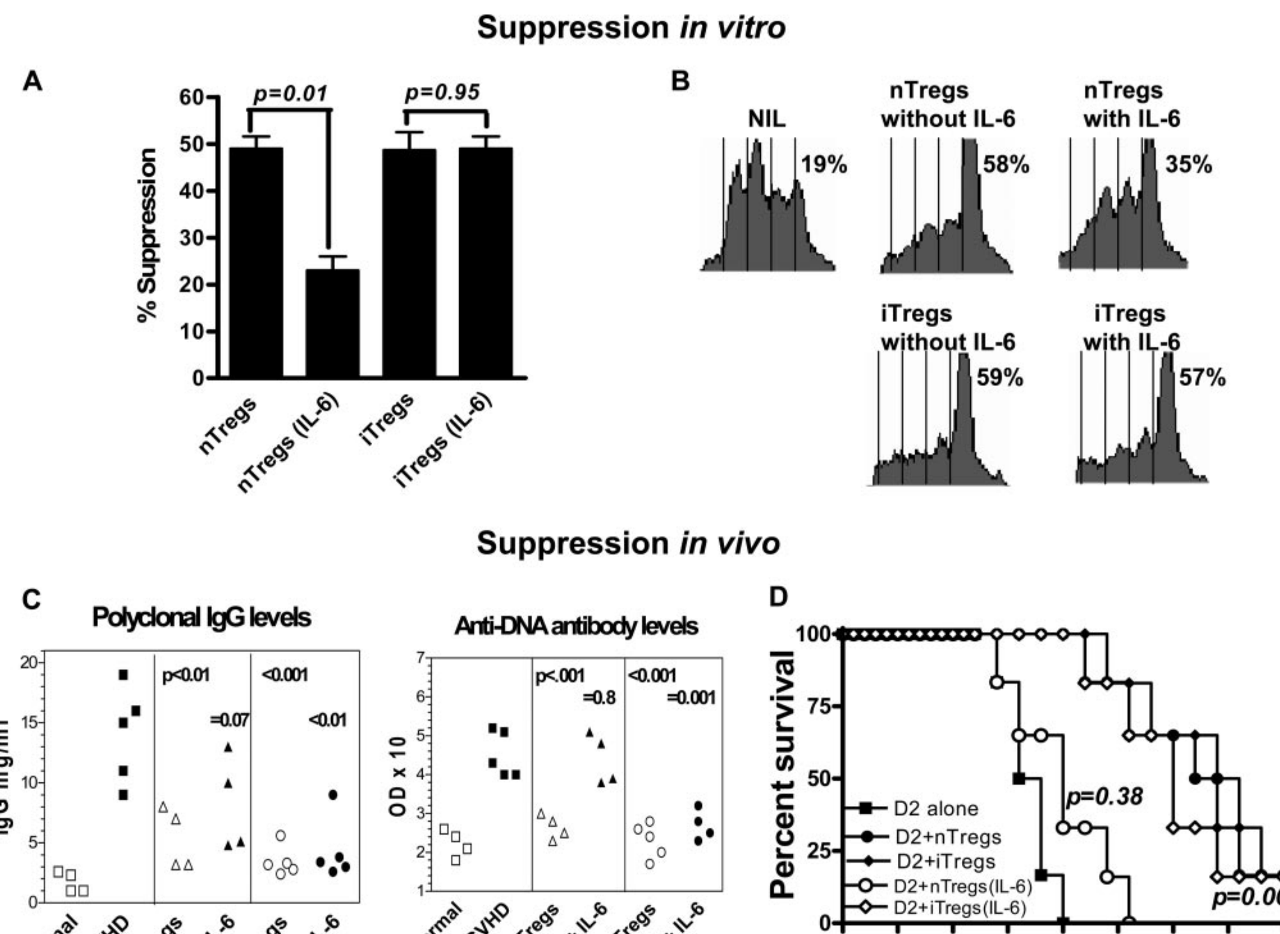

FIGURE 2. iTregs but not nTregs retain their suppressive phenotype when stimulated with IL-6. *A*, Suppression in vitro of nTregs and iTregs stimulated with or without IL-6 (1:4 ratio) was assayed using soluble anti-CD3-stimulated cells and determined by inhibition of cycling CFSE-labeled cells (mean ± SEM of three independent experiments). *B*, A representative histogram of the suppressor assay. Baseline indicates no added conditioned Tregs. The percentages of noncycling $CD4^+$ cells from one of three separate experiments are indicated. *C*, In vivo suppressor assay; GVHD with a lupus-like syndrome was produced by transfer of 80×10^6 DBA/2 spleen cells into D2 × B6F1 mice. Some groups also received 5×10^6 nTregs or iTregs that had been stimulated with or without IL-6. Twelve days after transfer, anti-DNA and IgG levels from serum were determined by ELISA. Values indicate the mean ± SEM of serum from four to five mice per group pooled from two independent experiments. The *p* value was calculated by Student's test and indicates significant differences between GVHD and addition of Tregs ($p < 0.05$). *D*, Kaplan Meier survival curves of the various groups of mice shown in *C* (D2, D2 × B6F1). The *p* values were determined by the log rank test using GraphPad4 PRISM software.

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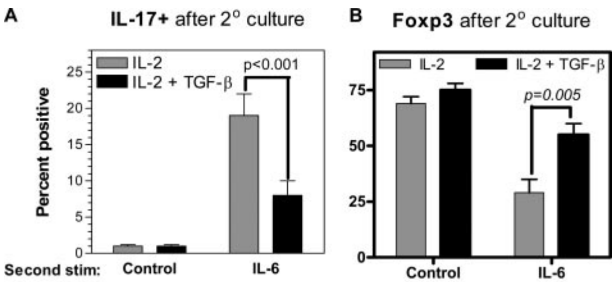
The ability of IL-6-treated nTregs to prevent autoimmunity in vivo was also markedly diminished. We used a chronic GVHD with a lupus-like syndrome model to investigate the effects of both Tregs before and after treatment with IL-6. Similar to alloantigen-specific Tregs (8), transfer of 5 million nTregs or polyclonal iTregs markedly blocked the increase in IgG production and suppressed anti-DNA autoantibody formation that occurs 2 wk after cell transfer (Fig. 2*C*). Whereas nTregs activated with IL-6 partially lost suppressive activity on IgG production and completely lost inhibitory activity on anti-dsDNA production, similarly treated iTregs maintained all suppressive effects. Although adoptive transfer of both polyclonal nTregs and iTregs significantly prolonged survival of the animals ($p < 0.002$), the protective effects of nTregs was almost completely lost after these cells were treated with IL-6. By contrast, after this treatment the protective effects of similarly treated iTregs were intact (Fig. 2*D*).

IL-2 and TGF-β stabilize the phenotype and functional activity of $Foxp3^+$ $CD4^+$ Tregs

We next asked whether the different effects of IL-6 on nTregs and iTregs were due to lineage characteristics or to cytokine effects on phenotypically and functionally similar populations. We considered that the lack of exposure of nTregs to IL-2 and TGF-β could account for the contrasting effects of IL-6 and found that this cytokine combination had dramatic effects on both IL-6 receptor expression and signaling. Priming nTregs with IL-2 and TGF-β significantly decreased the ability of IL-6 to down-regulate Foxp3 and convert the nTregs to Th17 cells (Fig. 3, *A* and *B*). Importantly, even though activated nTregs can produce TGF-β, pretreatment with IL-2 by itself could not control these effects of IL-6. Exogenous TGF-β was also necessary.

Although others have observed that TCR activation decreases IL-6 receptor expression (15), we observed that stimulation of



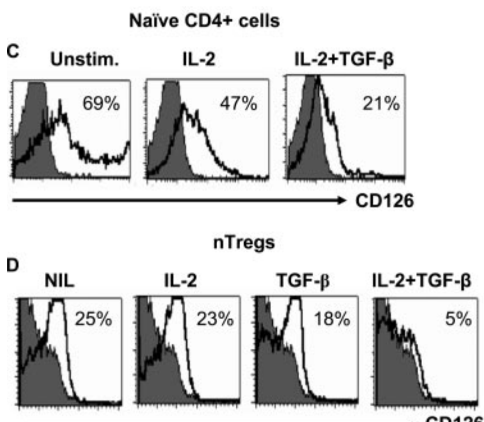


FIGURE 3. Treatment of nTregs with IL-2 and TGF-β prevents Th17 conversion and down-regulates IL-6R α-chain (CD126). *A* and *B*, nTregs were stimulated with anti-CD3/CD28-coated beads and IL-2 with or without TGF-β for 3 days and restimulated (Second stim.) with IL-6 as described in Fig. 1. The percentages of the intracellular IL-17 (*A*) and Foxp3 (*B*) expression on the $CD4^+CD25^+$ cells are shown. Values indicate the mean ± SEM of three separate experiments. The *p* value was calculated by Student's test and indicates significant differences between the treatment with or without TGF-β. *C*, Naive $CD4^+$ cells were unstimulated (Unstim.), stimulated with anti-CD3/CD28-coated beads plus IL-2, or stimulated with both IL-2 plus TGF-β to induce iTregs. Each population was then stained with anti-IL-6 Rα (solid line), and the isotype control IgG (shaded) and the overlaid histograms are shown. *D*, nTregs were similarly stimulated with anti-CD3/CD28 beads without cytokines (NIL), with IL-2 only, with TGF-β only, or with both cytokines for 3 days and CD126 expression was similarly determined as in *C*. The results of *C* and *D* are representative of three separate experiments.

naive $CD4^+$ cells with IL-2 and TGF-β to generate iTregs enhanced the loss of the α-chain (CD126) (Fig. 3*C*). Similarly, activation of nTregs with both IL-2 and TGF-β almost completely abolished CD126 expression whereas either of these cytokines added alone had minimal effects (Fig. 3*D*). These cytokines also down-regulated the IL-6R β-chain (CD130) expressed by nTregs (not shown).

Notwithstanding the inhibitory effects of IL-2 and TGF-β on CD126 expression, this result could not explain the different effects of IL-6 on nTregs and iTregs because both subsets expressed similar percentages of these receptors. Whereas IL-6 phosphorylates Stat3 and up-regulates Smad7 mRNA (16, 17), iTregs were completely resistant to these effects in contrast to nTregs (Fig. 4, *A* and *B*). However, once nTregs had been activated with IL-2 and TGF-β, IL-6 signaling by these cells also was markedly decreased (Fig. 4*A*). Thus, the combination of IL-2 and TGF-β inhibits IL-6R expression and signaling by both naive $CD4^+$ cells and mature nTregs.

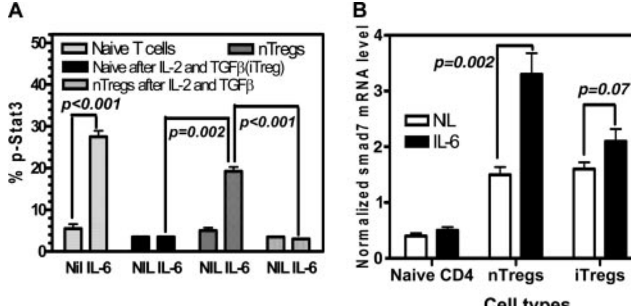


FIGURE 4. iTregs but not nTregs display decreased IL-6 signaling when stimulated with IL-6. Naive T cells, iTregs, and nTregs were stimulated as described in Fig. 1*A* without (Nil) or with IL-6. We also included nTregs pretreated with IL-2 and TGF-β. *A*, Intracellular phosphorylated STAT3 expression was determined by flow cytometry. *B*, Smad7 mRNA expression was determined by real-time PCR. Values indicate the mean ± SEM of three separate experiments. The *p* values were calculated by Student's *t* test and indicate significant effects of IL-6 on naive CD4 cells and nTregs that disappear after the cells are treated with IL-2 and TGF-β.

These studies further emphasize the importance of both IL-2 and TGF-β in maintaining immune tolerance (7). In addition to synergizing with TGF-β to induce Th17 cells, IL-6 inhibits Treg function (18) and Treg expansion (19) and induces Smad7, which inhibits TGF-β activity (17). In autoimmune diseases such as system lupus erythematous, IL-6 production is increased and production of IL-2 and TGF-β is decreased (20). The numbers of $Foxp3^+$ Tregs may be adequate in system lupus erythematous and other autoimmune diseases, but these cells are unable to overcome the inhibitory effects of IL-6 because of insufficient IL-2 and biologically active TGF-β. Others have reported a role for IL-2 in controlling Th17 differentiation. One group has recently reported that IL-2 signaling via Stat 5 constrains IL-17 production (21). Another group reported a model of T cell-mediated autoimmune disease resembling GVHD where IL17 was the principal cytokine responsible for tissue inflammation and IL-2 ameliorated this effect (22). Others have reported that iTregs induced by IL-2 and TGF-β have protective effects in autoimmune gastritis (23), experimental myasthenia gravis (24), and allergic encephalomyelitis (25). Previously we reported that iTregs could increase the survival of mice with established chronic GVHD (8). The transfer of these iTregs may have favorable therapeutic effect because of their resistance to the proinflammatory effects of IL-6. Thus iTregs may have special therapeutic potential in immune-mediated chronic inflammatory diseases characterized by high levels of IL-6. Because IL-6 and TGF-β may have different effects on human T cells than mouse T cells, these results must be interpreted with caution.

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Disclosures

D. A. Horwitz is a consultant for BD Biosciences, San Jose, CA. The remaining authors have no conflicting financial interests.

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