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CUTTING EDGE

Cutting Edge: IL-2 Is Essential for TGF-β-Mediated Induction of Foxp3$^+$ T Regulatory Cells

Todd S. Davidson, Richard J. DiPaolo, John Andersson, and Ethan M. Shevach[1]

***TGF-β is a pluripotent cytokine that is capable of inducing the expression of Foxp3 in naive T lymphocytes. TGF-β-induced cells are phenotypically similar to thymic-derived regulatory T cells in that they are anergic and suppressive. We have examined the cytokine and costimulatory molecule requirements for TGF-β-mediated induction and maintenance of Foxp3 by CD4$^+$Foxp3$^-$ cells. IL-2 plays a non-redundant role in TGF-β-induced Foxp3 expression. Other common γ-chain-utilizing cytokines were unable to induce Foxp3 expression in IL-2-deficient T cells. The role of CD28 in the induction of Foxp3 was solely related to its capacity to enhance the endogenous production of IL-2. Foxp3 expression was stable in vitro and in vivo in the absence of IL-2. As TGF-β-induced T regulatory cells can be easily grown in vitro, they may prove useful for the treatment of autoimmune diseases, for the prevention of graft rejection, and graft versus host disease. The Journal of Immunology,* 2007, 178: 4022–4026.**

Regulatory T cells (Treg)[2] are responsible for limiting most immune responses and inhibiting the induction of autoimmunity (1). Although Treg were originally identified as CD4$^+$CD25$^+$ T cells, more recent studies have defined naturally occurring Treg (nTreg) as expressing the forkhead family transcription factor, Foxp3. Although Foxp3 is necessary for the development of nTreg in the thymus, it may not be sufficient. A number of studies have examined the requirement for cytokines for nTreg development. Engagement of CD28 is critical for the generation of Treg (2), but IL-2 does not appear to be required (3) for the generation of Foxp3$^+$ nTreg in vivo. The role of IL-2 in the maintenance and function of nTreg in the periphery still remains to be clarified (3–6). Although TGF-β-deficient mice develop an autoimmune syndrome (7) similar to that of Foxp3$^{-/-}$ mice, nTreg develop normally in the thymus in the absence of TGF-β, but their maintenance and function in the periphery may be compromised (8).

In contrast to the requirement for the development of Foxp3$^+$ nTreg in the thymus, recent reports (9, 10) have demonstrated that peripheral CD4$^+$Foxp3$^-$ T cells can be induced in vitro to express Foxp3 in the presence of TGF-β. These induced cells, termed iTregs, are functionally similar to nTregs in that they are anergic, suppressive, and capable of inhibiting disease in vivo. In this study, we have examined the cytokine and costimulatory molecule requirements for the TGF-β-mediated induction and maintenance of Foxp3 by CD4$^+$CD25$^-$ cells. Although signaling via CD28 is not required, IL-2 plays a critical role in the induction, but not the maintenance of Foxp3 expression in vitro and in vivo.

Materials and Methods

Mice

B10.A 5CC7 TCR-Tg Rag2$^{-/-}$ and B10.A 5CC7 TCR-Tg Rag2$^{-/-}$ IL-2$^{-/-}$ mice were obtained from Taconic Farms. C57BL/6 mice were obtained from Jackson ImmunoResearch Laboratories. Mice were housed under specific pathogen free conditions in the National Institute of Allergy and Infectious Disease animal facility and used at 6- to 10-weeks of age.

Cytokines, reagents, and antibodies

Recombinant human TGF-β was obtained from R&D Systems and used at 5 ng/ml. Human recombinant IL-2 was purchased from Peprotech and used at 100 U/ml. All other cytokines were purchased from R&D Systems and used at 10 ng/ml. Abs to mouse CD3, CD28, and CD4-FITC were obtained from BD Biosciences. The anti-Foxp3 Alexa Fluor 647 Ab clone 150D was obtained from BioLegend. The anti-IFN-γ Ab XMG1.2, anti-IL-4 Ab 11B11, and anti-IL-2 Ab S4B6 were generated in our laboratory and used as ascites. CFSE was obtained from Molecular Probes and used at a final concentration of 1 μM.

Cell purification and media

Lymph nodes and spleens were harvested from mice that had been sacrificed by CO_2 asphyxiation. They were mechanically dissociated and strained through a 40-μm nylon mesh to produce a single cell suspension. RBC were lysed using ACK lysis buffer (BioWhittaker). The cells were labeled with CD4 beads (Miltenyi Biotec) and positively selected using an AutoMacs (Miltenyi Biotec). The cells were washed with PBS then resuspended in RPMI 1640 (BioSource) medium that contained 10% heat-inactivated FBS, 2-ME, HEPES, non-essential amino acids, penicillin, streptomycin, and L-glutamine.

Th neutral, Th1 and Th2 differentiation

Purified CD4$^+$ T cells were cultured in 24-well plates (Corning Costar) with plate-bound anti-CD3 and anti-CD28 Abs. For Th1 differentiation, the cells were cultured in the presence of 10 ng/ml IL-12 and 10 μg/ml anti-IL-4. For Th2 differentiation the cells were cultured with 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ. Th neutral cells were generated by culture without any Abs or exogenous cytokines. The cells were differentiated for 1 week. Differentiation was assayed by restimulating the cells with PMA/ionomycin followed by intracellular staining for IFN-γ and IL-4.

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[2] Abbreviations used in this paper: Treg, regulatory T cell; nTreg, naturally occurring Treg; BMDC, bone marrow-derived dendritic cell.

Cell culture and FACS analysis

Purified CD4$^+$ T cells were cultured in 24-well plates (Corning Costar) in the presence of plate-bound anti-CD3 and anti-CD28 Abs. Unless otherwise noted, all wells were coated with 2 μg/well of each Ab for 2 h at 37°C in PBS. Cells were cultured at an initial density of 2.5–5 $\times$ 10^5 cells per well. At the end of the culture period, the cells were harvested, washed with PBS and stained with CD4-FITC in PBS containing 0.5% BSA (PBS-BSA) at 4°C for 15 min. The cells were then washed with PBS-BSA and permeabilized overnight in Fix/perm buffer (eBioscience). The cells were then washed once in PBS-BSA, then once again in permeabilization buffer (eBioscience). Nonspecific binding sites were blocked with a 1/100 dilution of rat Ig (Jackson ImmunoResearch Laboratories) for 15 min at 4°C and then the cells were stained with the anti-Foxp3-AF647 clone 150D at a final dilution of 1/150 for 45 min at 4°C. The cells were then washed, resuspended in PBS, and analyzed on a FACSCalibur (BD Biosciences).

Proliferation assays

CD4$^+$CD25$^-$ responder cells were purified from lymph nodes by first depleting the CD25+ fraction, then positively selecting the CD4$^+$ fraction with magnetic beads on an AutoMacs (Miltenyi Biotec). T-depleted spleen cells were obtained by depletion of Thy1.2$^+$ cells with magnetic beads. Responder cells (5 $\times$ 10^4) were cultured with an equal number of T-depleted spleen cells as APC in the presence of 0.5 μg/ml anti-CD3 (clone 2C11) in 96-well microtiter plates for 72 h. [^{3}H]TdR (1 μCi/well) was added for the final 6 h. The cultures were then harvested and [^{3}H]TdR incorporation measured by scintillation counting.

Results and Discussion

The ability of TGF-β to induce Foxp3 expression in CD4$^+$CD25$^-$Foxp3$^-$ cells was evaluated by stimulating a homogenous population of CFSE-labeled CD4$^+$Foxp3$^-$ T cells from a TCR transgenic mouse on a Rag$^{-/-}$ background with plate-bound anti-CD3 and anti-CD28 in the presence of TGF-β in medium containing IL-2. TCR transgenic Rag$^{-/-}$ animals do not contain an endogenous population of Foxp3+ cells, thus any Foxp3 expression detected as a result of TGF-β treatment would result from de novo expression. At 48 h after stimulation, nearly 50% of the TGF-β-treated cells were Foxp3$^+$, whereas <1% of the cells stimulated in the absence of the cytokine were Foxp3$^+$ (Fig. 1*A*). The number of Foxp3-expressing cells continued to increase to 73% by 72 h, and reached a maximum of 83% at 96 h. In contrast, <1% of the cells not treated with TGF-β were Foxp3$^+$ by 72 h, and only 2% of the cells stained positive for Foxp3 at 96 h. TGF-β treatment did not affect the ability of the cells to proliferate as indicated by identical CFSE dilution in the treated and untreated groups. These results are consistent with previous data that demonstrated that TGF-β is capable of inhibiting T cell proliferation when the cells are stimulated with anti-CD3 alone, but that this inhibition is overcome in the presence of CD28 costimulation (11).

To dissect the contribution of IL-2 and anti-CD28 to the induction of Foxp3, the CD4$^+$Foxp3$^-$ T cells were stimulated in the absence of both anti-CD28 and IL-2, with anti-CD28 alone, or IL-2 alone. In the absence of both anti-CD28 and exogenous IL-2, 28% of the cells expressed Foxp3 at 48 h (Fig. 1*B*). In the presence of 100 U/ml of added IL-2 alone, the

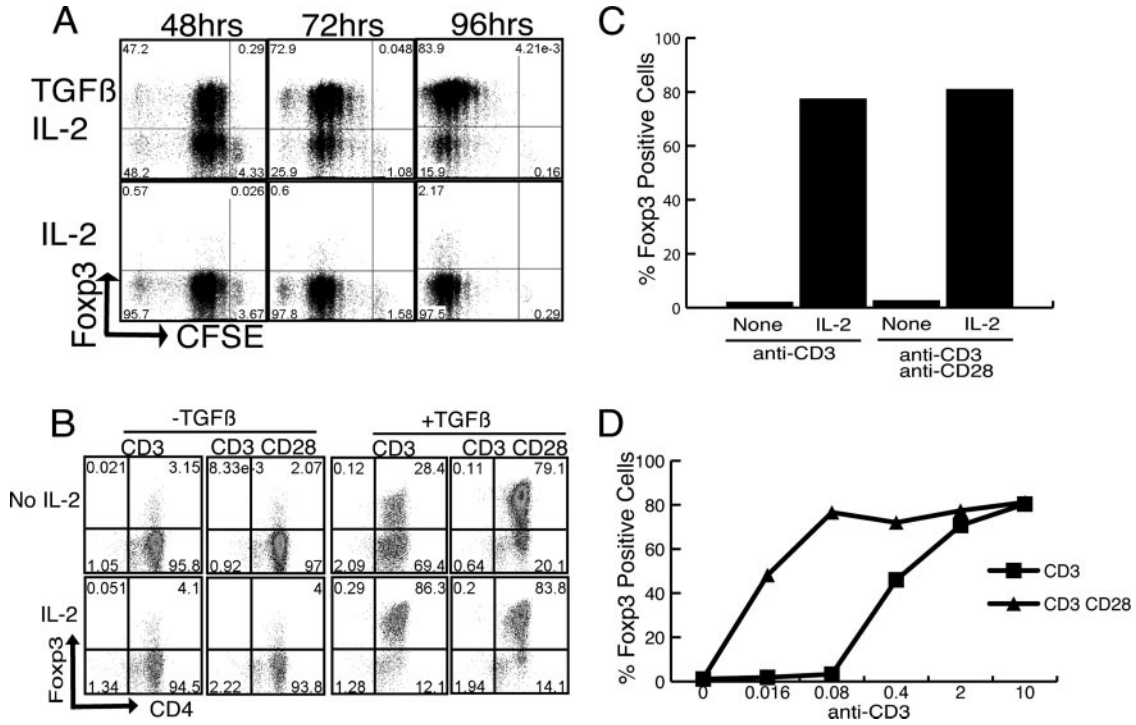

FIGURE 1. TGF-β induces Foxp3 expression in an IL-2-dependent manner. *A*, 5CC7 TCR-Tg Rag$^{-/-}$ CD4$^+$ T cells were purified, labeled with CFSE, and stimulated with plate-bound anti-CD3 and anti-CD28 Abs in the presence of exogenous IL-2 either with (*top panels*) or without (*bottom panels*) 5 ng/ml TGF-β. At the indicated time points, cells were harvested and analyzed for Foxp3 expression and CFSE dilution by flow cytometry. *B*, CD4$^+$5CC7 TCR-Tg Rag$^{-/-}$ T cells were cultured on plate-bound anti-CD3 (2 μg/well) with and without the addition of plate-bound anti-CD28 (2 μg/well) in medium that contained either no exogenous cytokines or medium that contained 100 U/ml IL-2. The cells were cultured for 48 h and analyzed for the expression of CD4 and Foxp3 by flow cytometry. *C*, Purified CD4$^+$5CC7 TCR-Tg Rag$^{-/-}$IL-2$^{-/-}$ T cells were stimulated with plate-bound anti-CD3 or anti-CD3 plus anti-CD28 in the presence or absence of exogenous IL-2. At 72 h, the cells were stained for Foxp3 expression. The percentage of live CD4$^+$Foxp3$^+$ cells is displayed on the *y*-axis. *D*, Purified CD4$^+$5CC7 TCR-Tg Rag$^{-/-}$ T cells were cultured in medium that contained no exogenous cytokines with the indicated concentrations of anti-CD3 (μg/well). Plate-bound anti-CD28 was used at a concentration of 2 μg/well in all indicated cultures. The cells were cultured for 72 h, and analyzed for expression of Foxp3 by flow cytometry. The percentage of Foxp3$^+$ cells is displayed on the *y*-axis.

percentage of Foxp3$^+$ cells increased to 86%. Similarly, CD28 costimulation in the absence of exogenous IL-2 resulted in induction of 79% Foxp3$^+$ cells. The combination of the two resulted in percentages of Foxp3$^+$ cells similar to either stimulus alone. Thus, for maximal induction of Foxp3 expression, exogenous IL-2 is able to eliminate the need for CD28 costimulation, and CD28 costimulation is able to compensate for a lack of exogenous IL-2. To determine whether the effects of anti-CD28 costimulation were mediated by induction of endogenous IL-2 production, we stimulated CD4$^+$Foxp3$^-$ T cells from TCR transgenic Rag$^{-/-}$ IL-2$^{-/-}$ animals with plate-bound anti-CD3 and TGF-β in the presence or absence of CD28 stimulation. In the absence of exogenous IL-2, <5% of the IL-2$^{-/-}$ cells expressed Foxp3 after 72 h of culture even when stimulated with anti-CD28 (Fig. 1*C*). When IL-2 was added nearly 80% of the cells expressed Foxp3 in both the CD28-stimulated and unstimulated conditions. Thus, in the absence of IL-2, TGF-β is unable to induce Foxp3 expression even in the presence of CD28 signaling. Neutralization of endogenously produced IL-2 by IL-2-sufficient CD4$^+$ cells with anti-IL-2 completely inhibited TGF-β-induced Foxp3, indicating that the result obtained with the IL-2$^{-/-}$ cells was not due to an unanticipated defect in this population. Foxp3 expression was observed in a small population of cells at 48 h that had not undergone cell division, and induction of Foxp3 in this subpopulation was also inhibited by anti-IL-2 (data not shown) indicating that IL-2 is not merely expanding a population induced by TGF-β alone.

We also evaluated the role of the strength of the TCR signal required to up-regulate Foxp3 in the presence of TGF-β. CD4$^+$Foxp3$^-$ T cells were stimulated with varying concentrations of plate-bound anti-CD3 without any exogenous IL-2. The role of CD28 costimulation was also investigated by adding a fixed concentration of plate-bound anti-CD28. In the absence of CD28 costimulation, Foxp3 expression was maximal at 2 μg/well anti-CD3, whereas significant Foxp3 levels were not detectable below 0.4 μg/well of anti-CD3 (Fig. 1*D*). In the presence of CD28 costimulation, maximal Foxp3 expression was seen at 25-fold lower concentrations of anti-CD3 stimulation. In separate experiments, we directly compared the ability of TGF-β to induce Foxp3 when T cells were stimulated either by plate-bound anti-CD3 and anti-CD28 Abs, soluble anti-CD3 anti-CD28 in the presence of bone marrow-derived dendritic cells (BMDC), or BMDC with cognate Ag. All conditions induced identical levels of Foxp3 expression (data not shown).

We next determined whether these TGF-β-treated Foxp3-expressing cells were capable of suppressing the proliferation of a naive population of effector T cells. CD4$^+$CD8$^-$CD25$^-$ thymocytes were stimulated in the presence or absence of TGF-β with exogenous IL-2, anti-CD3, and T-depleted spleen cells for 5 days. The cells were then washed and transferred to medium containing IL-2 for 72 h. The suppressive activity of the cells was then assayed by co-culturing them at various ratios with CD4$^+$CD25$^-$ responder cells, anti-CD3, and T-depleted spleen cells as APC. Proliferation was assessed at 72 h by thymidine incorporation. In the presence of the TGF-β-treated cells, proliferation was potently reduced, whereas the non-TGF-β-treated cells actually enhanced proliferation (Fig. 2). Thus, TGF-β treatment of CD4$^+$CD25$^-$Foxp3$^-$ T cells results in Foxp3 expression and a suppressive phenotype.

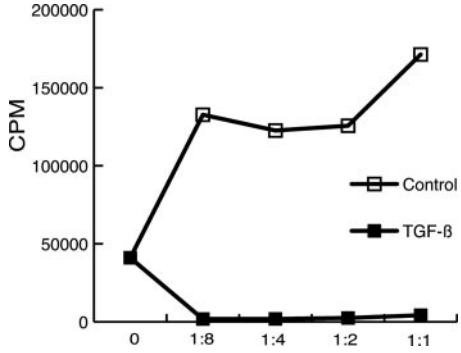

FIGURE 2. TGF-β-induced Foxp3$^+$ cells are suppressive. CD4$^+$CD25$^-$CD8$^-$ thymocytes from BALB/c mice were stimulated in the presence or absence of TGF-β with IL-2, anti-CD3 and T-depleted splenocytes (TdS) for 5 days. The cells were then washed and placed in medium containing IL-2 with no further TCR stimulus for an additional 72 h. The TGF-β-treated cells used in this experiment were ~90% positive for Foxp3. The cells were then cultured with the indicated ratios of induced cells to CD4$^+$CD25$^-$ responder cells as described in *Materials and Methods*. Results are expressed as cpm.

The ability of TGF-β to induce Foxp3 expression and endow the cell with a suppressive phenotype immediately suggests a potential therapeutic intervention whereby Ag-specific, autoaggressive effector T cells could be transformed ex vivo into Ag-specific suppressors. However, for such a therapy to be practical, it would be necessary to induce a Foxp3$^+$ suppressive phenotype in differentiated, activated effector T cells. To determine whether Foxp3 expression could be induced in previously activated effector T cells, we cultured CD4$^+$Foxp3$^-$ T cells under either Th1, Th2, or Th-neutral conditions in vitro for 1 week and then restimulated the cultures in the presence of TGF-β for 72 h (Fig. 3). Only 15% of the cells activated under Th-neutral conditions could be induced to express Foxp3 in the presence of TGF-β. Both Th1- and Th2-differentiated cells were completely refractory to TGF-β-induced Foxp3 expression. Although in vitro differentiated cells may not represent a true memory population, these results do strongly indicate that recently activated T cells with different cytokine secretion profiles cannot be induced to express Foxp3 by re-stimulation in the presence of TGF-β.

The IL-2R utilizes the common γ-chain (γc) to transduce signals. As the γc is shared by receptors for IL-4, IL-7, IL-9,

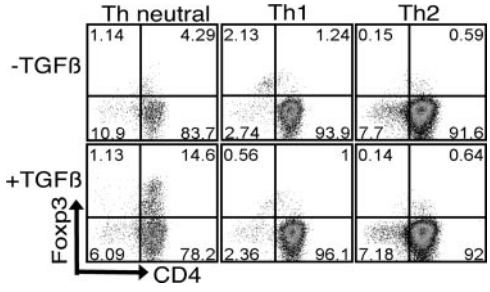

FIGURE 3. Foxp3 expression cannot be induced in previously differentiated cells. CD4$^+$5CC7 TCR-Tg Rag$^{-/-}$ T cells were purified and differentiated in vitro for 1 wk under Th-neutral, Th1, or Th2 conditions as described in *Materials and Methods*. The cells were then restimulated for 48 h with or without 5 ng/ml TGF-β on 2 μg/well each plate-bound anti-CD3 and anti-CD28 Abs in the presence of 100 U/ml IL-2. The cells were then analyzed for CD4 and Foxp3 expression by flow cytometry.

IL-15, and IL-21, we evaluated whether one of these, or potentially other cytokines, could substitute for IL-2 in TGF-β-mediated Foxp3 induction. No other cytokines utilizing γc were able to induce Foxp3 expression in the IL-$2^{-/-}$ T cells (Fig. 4*A*). Induction of Foxp3 was also not observed with IL-6, IL-12, IL-18 (Fig. 4*A*), or IL-10 (data not shown).

To address the important issue of the stability of TGF-β-induced Foxp3 expression, $CD4^+$ $Foxp3^-$ cells were stimulated in the presence of TGF-β, labeled with CFSE, and transferred into wild-type and IL-$2^{-/-}$ recipients. Foxp3 expression on $CD4^+CFSE^+$ cells was analyzed after 7 days (Fig. 4*B*). The great majority of the transferred cells retained a high level of Foxp3 expression. The level of expression and the percentage of cells expressing Foxp3 were comparable to the starting population that was injected (data not shown). The recovery of the transferred cells (both percentages and absolute numbers) from the IL-$2^{-/-}$ recipients and their level of Foxp3 expression were identical to that of cells from the IL-2 sufficient hosts. Two major points can be drawn from these data. First, Foxp3 expression is a stable phenotype that is maintained upon adoptive transfer. Second, although required for its induction, IL-2 is dispensable in vivo, at least for the period of time studied, to maintain the expression of Foxp3. Similar results were observed when the TGF-β-induced cells were washed and put back into culture with various cytokines for 72 h in the absence of additional TCR stimulus or costimulation. All cytokines tested (IL-2, -4, -6, -7, -9, -12, -15, -18, -21) appeared to be fairly equivalent in terms of allowing maintenance of Foxp3 expression although they did differ in their ability to maintain cell viability (data not shown).

These results confirm and extend earlier studies (9, 10, 12) on the capacity of TGF-β to induce $Foxp3^+$ T cells. In many respects, these iTreg behave in an identical fashion to thymic-derived nTreg in vitro and in vivo in terms of their suppressive activity. However, our results demonstrate several major differences in the requirements for IL-2 and CD28 costimulation in the induction of Foxp3 expression in nTreg compared with iTreg. The studies of Fontenot et al. (3) using mice containing a $Foxp3^{gfp}$ "knockin allele" clearly demonstrate that IL-2 is not required for the development of nTreg in the thymus although it likely plays a role in their maintenance and metabolic state in the periphery. In contrast, our studies demonstrate an absolute requirement for IL-2 in concert with TGF-β for induction of $Foxp3^+$ iTreg in the periphery. We have not excluded a role for cytokines other than the ones we have tested, but other cytokines that utilize the γc-chain as part of their receptor complexes were completely ineffective. Once induced, Foxp3 expression was stable both in vitro and in vivo in the absence of IL-2. It remains possible that IL-2 is still required for the long-term maintenance of $Foxp3^+$ iTreg, but we have not conducted the in vivo studies for longer than 2 weeks in IL-$2^{-/-}$ mice although it is stable for 50 days in IL-2-sufficient mice (R. J. DiPaolo, T. S. Davidson, J. Andersson, E. M. Shevach, manuscript in preparation). A second major difference between nTreg and iTreg is that the development of nTreg required CD28-mediating signaling in addition to those required for IL-2 production (2), whereas the role of CD28 in the induction of iTreg appears to be solely related to its capacity to enhance the endogenous production of IL-2.

One major question that remains unresolved by these studies is whether and under what conditions iTreg are generated in vivo. It remains unknown whether any of the $Foxp3^+$ T cells in mouse or more importantly in man are derived in the periphery by this TGF-β-dependent induction pathway. It is likely that inflammatory infiltrates would contain sufficient quantities of both TGF-β and IL-2, so this induction pathway should be operative in vivo. However, such infiltrates would also contain proinflammatory cytokines, such as IL-6, that could subvert the induction of iTreg and result in the induction of IL-17 producing T cells (12). The therapeutic potential of TGF-β iTreg also remains to be determined. Our results indicate that T cells recently activated in vitro cannot be induced to express Foxp3. As most autoantigen-specific cells that might be isolated from patients are also likely to be memory T cells, it may be difficult to convert them to iTreg expressing Foxp3. On the other hand, alloantigen-specific TGF-β iTreg might be readily induced

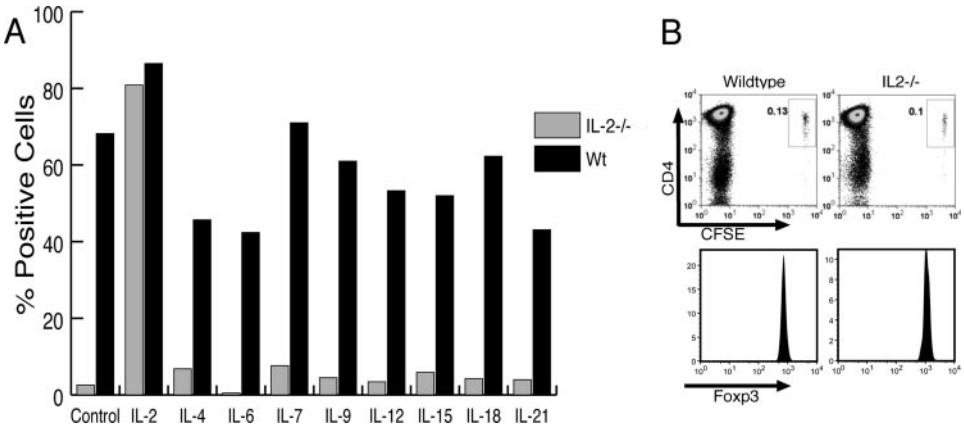

FIGURE 4. IL-2 is non-redundant in allowing TGF-β-mediated Foxp3 expression but is not required for maintenance of Foxp3 expression in vivo. *A*, $CD4^+$5CC7 TCR-Tg $Rag^{-/-}$ T cells on either an IL-2 sufficient (here referred to as WT) or IL-$2^{-/-}$ background were purified and stimulated in the presence of the indicated cytokines on plate-bound anti-CD3 and anti-CD28 Abs with 5 ng/ml TGF-β for 72 h. The control group is no cytokine in addition to TGF-β. The cells were then harvested and analyzed for the expression of Foxp3 by flow cytometry. The percentage of $Foxp3^+$ cells is displayed on the *y*-axis. *B*, $CD4^+$5CC7 TCR-Tg $Rag^{-/-}$ T cells were purified and stimulated on plate-bound anti-CD3 and anti-CD28 Abs in the presence TGF-β and 100 U/ml exogenous IL-2 for 1 week. One million cells were labeled with CFSE and then transferred into either 5CC7 TCR-Tg $Rag^{-/-}$ or 5CC7 TCR-Tg $Rag^{-/-}$ IL-$2^{-/-}$ recipients. Seven days later, the animals were sacrificed, lymph nodes collected and stained with allophycocyanin-conjugated anti-CD4 and PE-conjugated anti-Foxp3 and analyzed by flow cytometry. Foxp3 expression by $CD4^+CFSE^+$ cells is displayed in the histogram.

from naive precursors and should prove useful for the treatment of graft rejection and graft-vs-host disease.

Disclosures

The authors have no financial conflict of interest.

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