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IL-2 Is Essential for TGF- β to Convert Naive CD4⁺CD25⁻ Cells to CD25⁺Foxp3⁺ Regulatory T Cells and for Expansion of These Cells¹

Song Guo Zheng, Juhua Wang, Pu Wang,² J. Dixon Gray, and David A. Horwitz³

IL-2 and TGF- β both have important roles in the induction and maintenance of immunologic tolerance, but whether these cytokines act separately or together to achieve this effect is poorly understood. Although others have reported that IL-2 can directly enhance forkhead box protein P3 (Foxp3) transcription factor expression by natural CD4⁺CD25⁺ regulatory T cells, in this study, we report that the role of IL-2 on the generation of peripheral regulatory CD4⁺ cells is indirect. Ab neutralization studies and experiments with IL-2-deficient mice have revealed that IL-2 is required for TGF- β to induce naive CD4⁺CD25⁻ cells to become CD25⁺ and express Foxp3, and develop the characteristic properties of CD4⁺CD25⁺ regulatory cells. This effect of IL-2 on the generation and expansion of these adaptive Foxp3⁺ regulatory cells is nonredundant, but IL-4, IL-7, and IL-15, other common γ -chain cytokines, could sustain Foxp3 expression. Because subjects with autoimmune diseases often have defects in the production of IL-2 and/or TGF- β , the generation of autologous T regulatory cells ex vivo with these cytokines for transfer in vivo may have considerable therapeutic potential. *The Journal of Immunology*, 2007, 178: 2018–2027.

The T cell response to self and environmental Ags is controlled by regulatory/suppressor cell subsets consisting of heterogeneous CD4⁺, CD8⁺, or NKT cell subsets. These regulatory T (Treg)⁴ cell subsets consist of naturally occurring, thymus-derived cells that react to self Ags (1, 2) and peripherally induced, adaptive Treg cells that primarily respond to environmental Ags (3). Natural CD4reg cells constitutively express the IL-2R α -chain (CD25) and express the forkhead box protein P3 (Foxp3) transcription factor that is required for their differentiation (4–6). Some mouse and human adaptive CD4⁺CD25⁺ Treg cells also express Foxp3 and have a phenotype and contact-dependent mechanism of action that closely resemble natural CD4reg cells (7–12).

Notwithstanding the similarities between natural and adaptive CD4⁺CD25⁺Foxp3⁺ Treg cells, there are significant differences in their generation. First, although TGF- β can induce peripheral CD4⁺CD25⁻ cells to become Treg cells, this cytokine is not required for the generation of the thymic-derived subset (13). Second, whereas positive CD28 costimulation is required for the generation of natural

CD4reg cells (14, 15), TGF- β -induced adaptive CD4reg cells require negative CTLA-4 costimulation (16). In this study, we report a third characteristic that distinguishes adaptive, TGF- β induced from natural CD4⁺CD25⁺ Treg cells.

TGF- β proteins have broad regulatory effects on T cell growth and differentiation that include inhibition of T cell proliferation and the generation of Th1 and Th2 cells (17). However, with sufficient stimulation to overcome inhibitory effects, TGF- β has a major positive role in the generation, function, and survival of adaptive CD4⁺ and CD8⁺ Treg subsets (7–12, 18–20). Although not required for generation of natural CD4⁺CD25⁺ Treg cells, evidence is accumulating that TGF- β proteins are essential for the function and survival of CD4reg cells (9, 19–22). Foxp3-expressing CD4⁺reg cells cannot survive in mice with genetically engineered T cells that are unresponsive to TGF- β (21, 22). Moreover, mice with T effector cells that cannot respond to TGF- β are resistant to Treg suppression (22). In addition, TGF- β also plays an important role in Treg homeostasis by maintaining the pool size of natural CD4⁺CD25⁺ cells in the periphery (21). Thus, it has become apparent that TGF- β has critical effects on both natural and adaptive CD4⁺CD25⁺ Treg cells.

Although IL-2 was first described as a T cell growth factor (23), evidence has accumulated that immunologic tolerance crucially depends upon this cytokine (24). This effect is principally due to the generation and maintenance of Treg cells rather than an alteration of central tolerance or an acceleration of activation-induced cell death (24, 25). Because natural CD4reg cells constitutively express IL-2R α , β , and γ -chains, it is not surprising that IL-2 signaling has been associated with Treg development, homeostasis, and function (1, 2). IL-2- and IL-2R-deficient mice develop extensive lymphadenopathy and systemic autoimmunity. Reconstitution of IL-2-deficient mice with a mixture of bone marrow cells from IL-2-sufficient and -insufficient mice corrected this pathology (24). Subsequently, others reported that IL-2 and IL-2R β gene deficiency result in a lack of CD4⁺CD25⁺ cells (25–27). Moreover, the adoptive transfer of wild-type CD4⁺CD25⁺ cells into IL-2R α - or IL-2R β -deficient mice prevents development of lymphadenopathy and autoimmunity (25, 27). Studies on the mechanism of IL-2 effects revealed that in the absence

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⁴ Abbreviations used in this paper: Treg, regulatory T; GITR, glucocorticoid-induced TNF receptor; HPRT, hypoxanthine guanine phosphoribosyl transferase; SLE, systemic lupus erythematosus.

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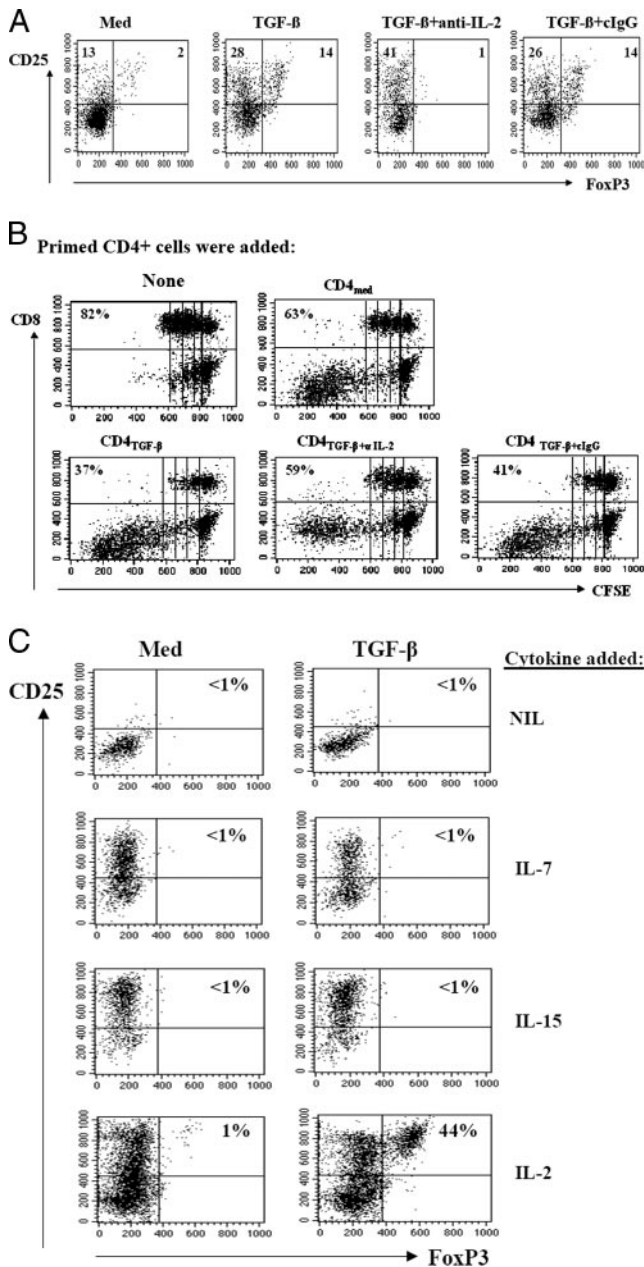


FIGURE 1. IL-2 is essential for TGF- β to induce CD4⁺ cells to express Foxp3 and develop the suppressive activity. *A*, Splenic CD4⁺CD25⁻ cells isolated from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28-coated beads (1:5) \pm TGF- β (2 ng/ml) with graded doses of anti-IL-2 or control IgG2a for 4 days. The concentration of Ab in this example was 2 μ g/ml. The cells were stained with Cyc-anti-CD4, PE-anti-CD25, and FITC-anti-Foxp3, and examined by flow cytometry. Scatter plots are gated on CD4 and are representative of four separate experiments. The percentage of activated cells expressing Foxp3 and CD25 is shown in each plot. *B*, Suppression was assayed using anti-CD3-stimulated cells as described in *Materials and Methods*. None indicates no added conditioned CD4⁺ cells. The ratio of CD4 conditioned cells to responder T cells was 1:4. The percentage of dividing CD8⁺ T cells from one of four independent experiments is shown with vertical lines indicating each cell division. *C*, Splenic naive CD4⁺CD25⁻CD62L⁺ cells from IL-2-deficient mice were stimulated with anti-CD3/CD28-coated beads \pm TGF- β \pm IL-7 (100 ng/ml), IL-15 (100 ng/ml), or IL-2 (20 U/ml) for 4 days. Staining was performed as described in Fig. 1*A*. Plots are gated on CD4, and the percentage of cells expressing both CD25 and Foxp3 is shown in the *upper right quadrant*. The example shown is representative of three separate experiments.

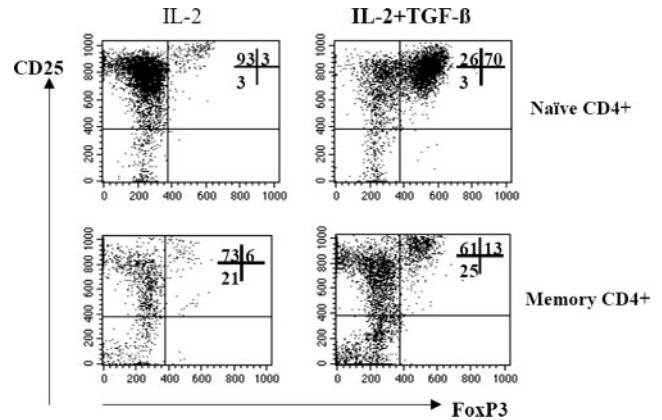


FIGURE 2. TGF- β predominantly induces naive CD4⁺ cells to express Foxp3. CD4⁺CD25⁻ cells isolated from C57BL/6 mice were further separated into CD62L⁺ (naive) and CD62L⁻ cells (memory) using MACS beads. Naive and memory CD4⁺CD25⁻ cells were stimulated with anti-CD3 and anti-CD28 beads \pm TGF- β in the presence of IL-2 (20 U/ml) for 4 days. Foxp3 and CD25 expression was determined by FACS analysis as described in Fig. 1*A*. The percentage of single- and double-positive cells in each quadrant is shown in the *upper right quadrant* and is representative of four independent experiments.

of this cytokine, Treg cells cannot survive or expand their numbers in the thymus and the periphery (28–33), and cannot suppress T cell proliferation (34, 35). From these data, it was concluded that IL-2 has essential, nonredundant function in the development, homeostasis, and function of Treg cells (24).

Whether IL-2 is responsible for the generation of all CD4⁺CD25⁺Foxp3⁺ Treg cells has become controversial. With the recent ability to identify Foxp3⁺ T cells, several groups have reported that IL-2 is dispensable for thymic induction of CD4⁺CD25⁺ Treg cells (26, 30, 35–37). Whether this is also the case for peripherally induced Treg is not known. For the past decade, we have been using IL-2 to overcome the suppressive effects of TGF- β to generate CD4⁺ and CD8⁺ Treg cells (18–20, 38, 39). To learn whether IL-2 is required for this purpose, we have neutralized IL-2 in studies with conventional mice, and studied naive CD4⁺ cells from IL-2-deficient mice. We report that only IL-2 can enable TGF- β to induce Foxp3 expression in T cells. Other cytokines that also signal through the β and/or common γ -chain of the IL-2R lacked this property. Finally, although IL-7 and IL-15 could substitute for IL-2 in sustaining Foxp3 expressed by these adaptive CD4⁺ reg cells, the presence of TGF- β was also required for this effect.

Materials and Methods

Mice

C57BL/6 and IL-2 knockout mice were purchased from The Jackson Laboratory. Some IL-2 knockout mice were provided by Dr. M. A. Farrar (University of Minnesota, Minneapolis, MN). Sex-matched mice aged 6–12 wk were used in all experiments. 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.

Reagents and Abs

PE-, FITC-, or CyChrome-conjugated anti-CD8 (53-6.7), CD11b (M1/70), B220 (RA36B2), CTLA-4 (UC10-4B9), CD62L (MEL-14), and respective matched control IgG Abs were purchased from BD Pharmingen. Conjugated anti-CD3 (17A2), CD4 (GK1.5), CD25 (PC61.5), CD103 (2E7), CD122 (TM-b1), IL-2 (JES6-5H4), and matched control IgG Abs were obtained from eBioscience. The FITC-conjugated anti-mouse/rat Foxp3 staining kit (FJK-16s) was purchased from eBioscience. TGF- β 1, biotin-conjugated anti-GITR (glucocorticoid-induced TNF receptor), and isotype

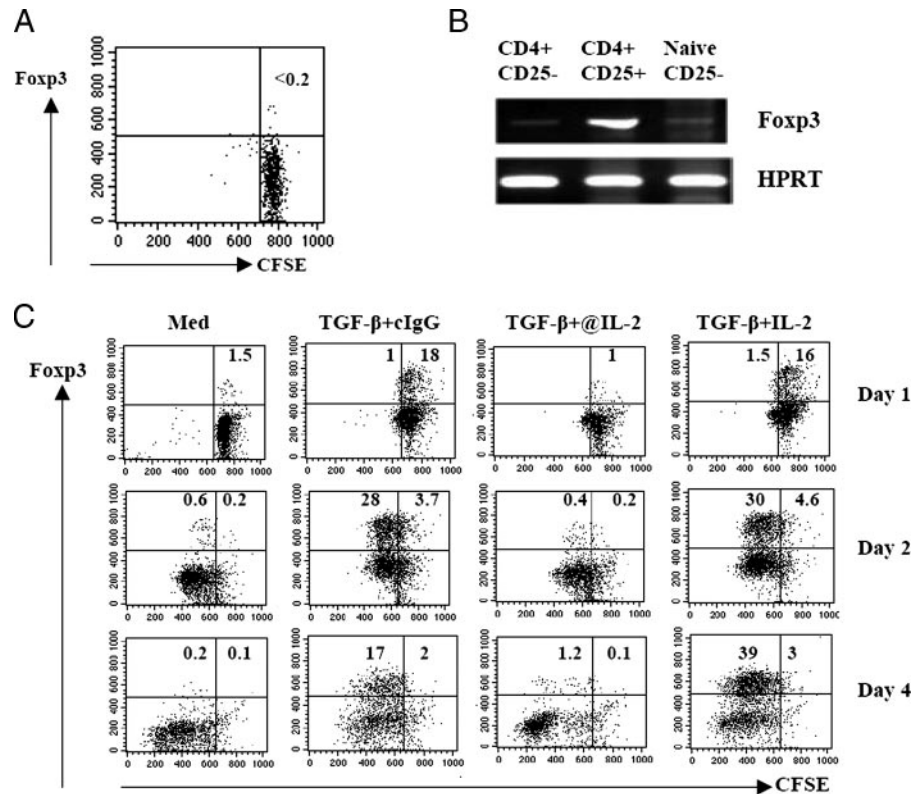


FIGURE 3. IL-2 is required for TGF- β to induce Foxp3 and for the expansion of these CD4⁺ cells. *A* and *B*, Foxp3 protein and mRNA was barely detectable in the starting population, CD4⁺CD62L⁺CD25⁻ cells as described in Fig. 2. *C* and *D*, Naive CD4⁺CD25⁻ cells were labeled with CFSE (1 μ M) and stimulated with anti-CD3/CD28-coated beads in the presence or absence of anti-IL-2 (1 μ g/ml), control IgG2a (1 μ g/ml), or exogenous IL-2 (20 U/ml) for the days indicated. Percentages of CD4⁺Foxp3⁺ cells were determined by FACS analysis. *C*, Values show one of three independent experiments. *D*, Values indicate the mean \pm SEM of three separate experiments.

control mAb, and rIL-2, IL-4, IL-7, IL-10 and IL-15 were purchased from R&D Systems. Unconjugated anti-TGF- β (9016), anti-IL-2 (142206) Ab, and isotype control Abs (all obtained from R&D Systems) were used for the neutralization experiments. Anti-CD3 and anti-CD28-coated beads were a gift from Dr. C. June (University of Pennsylvania, Philadelphia, PA). TRIzol was purchased from Invitrogen Life Technologies. AIM-V serum-free medium (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 mM HEPES (all obtained from Invitrogen Life Technologies) was used for the generation of CD4⁺ Treg or control cells. RPMI 1640 medium supplemented as just described with 10% heat-inactivated FCS (HyClone Laboratories) was used for all other cultures.

Cell isolation and generation of Treg cells

T cells were prepared from spleen cells by collecting nylon wool column nonadherent cells as described previously (16). CD4⁺ T cells were isolated by negative selection. Briefly, T cells were labeled with PE-conjugated anti-CD8, anti-CD11b, and anti-B220 mAbs, incubated with anti-PE magnetic beads, and loaded onto MACS separation columns (Miltenyi Biotec). The CD4⁺ cells were further labeled with FITC-conjugated anti-CD25 mAb, and CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were obtained by cell sorting (purity >98%). To prepare naive and memory CD4⁺CD25⁻ cells, CD4⁺CD25⁻ cells were labeled with PE-conjugated anti-CD62L and positively selected by anti-PE magnetic beads (CD4⁺CD62L⁺CD25⁻ cells) and negatively selected (CD4⁺CD62L⁻CD25⁻ cells). A total of 1.5×10^6 /ml CD4⁺, naive or memory CD4⁺ cells was stimulated with anti-CD3/CD28-coated beads (1:1) \pm TGF- β (2 ng/ml) \pm IL-2 (20 U/ml) in AIM-V serum-free medium for various days in 24-well plates (BD Labware). In some experiments, anti-IL-2 (2–10 μ g/ml), anti-TGF- β (10 μ g/ml), or sim-

ilar doses of control IgG were added to cultures. To learn whether other IL-2R β and common γ -chain cytokines can substitute for IL-2, IL-4 (10 ng/ml), IL-7 (100 ng/ml) or IL-15 (100 ng/ml) were added to cultures. We started with a range of doses and then chose an optimal dose of these cytokines to induce CD25 expression.

Assessment of Treg cell activity in vitro

These cells, generated as described above, were harvested and coated beads were removed by magnetic column. Various doses of CD4⁺ regulatory cells or control cells were added to fresh T cells (2×10^5) labeled with CFSE that were activated with anti-CD3 (0.25 μ g/ml) in the presence of irradiated APC (2×10^5) in 96-well flat-bottom microtiter plates. Proliferation was assessed by inhibition of percentages or total cycling CD8⁺ or CD4⁺ T cells as described previously (8).

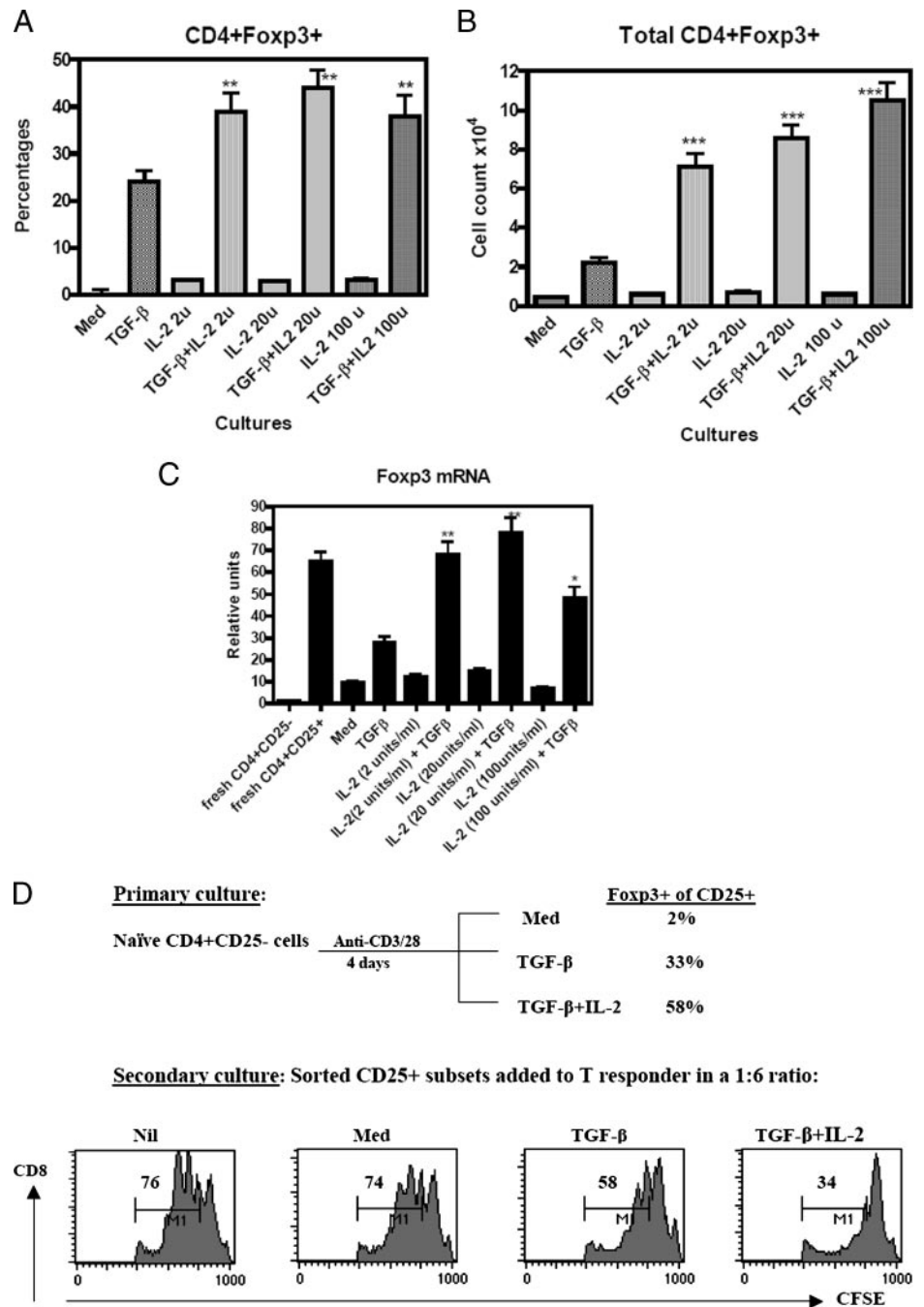
Intracellular staining

For detection of CTLA-4 by intracellular staining, CD4⁺ cells from the indicated different treatment groups were stained with anti-CD4 on the surface, then fixed and permeabilized with a kit purchased from Caltag Laboratories, and subsequently stained for CTLA-4. For intracellular staining of Foxp3, following various days of culture, CD4⁺ cells from the different treatment groups were harvested and stained according to the manufacturer's protocol.

RT-PCR for Foxp3 expression

Total RNA was extracted from cells using TRIzol reagent and used to determine the expression and relative level of the transcription factor Foxp3. First-strand cDNA was synthesized using Omniscript TR kit

FIGURE 4. Exogenous low-dose IL-2 enhances the ability of TGF- β to induce CD4⁺CD25⁺Foxp3⁺ cells with suppressive activities. *A* and *B*, FACS analysis of Foxp3 and CD25 expression by polyclonally activated naive CD4⁺CD25⁻ cells \pm TGF- β with or without IL-2 (2–100 U/ml) following culture for 4 days. Percentages (*A*) and total numbers (*B*) of CD4⁺Foxp3⁺ cells by polyclonally activated naive CD4⁺ cells stimulated with various doses of IL-2 \pm TGF- β . Values indicate the mean \pm SEM of five separate experiments. *C*, Foxp3 mRNA expression was determined by RT-PCR. The levels of Foxp3 were analyzed by semiquantitative RT-PCR after normalization of HPRT. Values indicate the mean \pm SEM of four independent experiments. Values of *p* were calculated by Student's *t* test and indicate significant effects of IL-2 on TGF- β -treated cells (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001). *D*, Primary culture: splenic naive CD4⁺CD25⁻ cells were polyclonally stimulated for 4 days. Some cultures contained TGF- β and IL-2 (20 U/ml) added alone or together. CD25⁺ subsets were then sorted from conditioned CD4⁺ cells primed with medium, TGF- β , or TGF- β plus IL-2 (20 U/ml), and Foxp3 expression on these CD25 subsets is shown. Secondary culture: purified CD25⁺ subsets were then added to T responder cells (1:6 ratio) and suppression was assayed as in Fig. 1*B* and Table I. The number indicates percentages of cycling CD8⁺ cells and is one of three independent experiments.



(Qiagen) with random hexamer primers (Invitrogen Life Technologies). Foxp3 and hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA was measured by semiquantitative RT-PCR using published primers (40). The relative expression of Foxp3 was determined by normalizing expression of each target to HPRT.

Statistical analysis

Statistical comparison between various groups was performed by the Student *t* test using GraphPad Prism software (GraphPad). Differences were considered significant when *p* values were <0.05.

Results

IL-2 is essential for TGF- β to induce CD4⁺ cells to express Foxp3 and develop suppressive activity

To learn whether IL-2 was required for TGF- β to induce CD4⁺ cells to express Foxp3, we polyclonally activated CD4⁺CD25⁻ cells with and without TGF- β . As we and others (7–11, 19, 20)

have noted previously, the addition of TGF- β markedly enhanced CD25 expression, and one third of these cells expressed Foxp3 (Fig. 1*A*). In the presence of a dose of anti-IL-2 Abs that did not inhibit TGF- β enhancement of CD25 expression, we observed that this partial neutralization of IL-2 almost completely abolished the ability of TGF- β to induce Foxp3. Moreover, the addition of this dose of anti-IL-2 to TCR-stimulated CD4⁺ cells with TGF- β also inhibited the generation of suppressive activity (Fig. 1*B*).

With this suggestive evidence, we next performed studies with CD4⁺ cells from IL-2^{-/-} mice. Naive CD62L⁺CD4⁺CD25⁻ T cells from these mice were stimulated with anti-CD3/28-coated beads \pm TGF- β . In this study, the addition of TGF- β did not enhance CD25 expression, and these TCR-stimulated cells did not express Foxp3 (Fig. 1*C*). Because these CD4⁺ cells did not exhibit CD25, the lack of Foxp3 induction could be explained by insufficient activation. Therefore, we used IL-15, a cytokine that uses

Table I. Relationship between Foxp3⁺ expression and suppressive activity^a

Cultures	% Foxp3/CD4	MFI of FoxP3 ⁺	% Suppression
Medium	2.1 ± 0.2		9.1 ± 1.2
IL-2	2.5 ± 0.2		7.6 ± 1.1
TGF- β	21 ± 1.9	516 ± 3.2	46.5 ± 4.6
TGF- β +IL-2	43 ± 3.5**	578 ± 4.4***	70 ± 4.2***

^a Values indicate the mean ± SEM of six independent experiments. Student's *t* test was used to compare the percentages of Foxp3⁺ cells, mean fluorescein intensity (MFI) expressed by CD4⁺ cells, and suppressive activity between CD4⁺ cells primed TGF- β alone and IL-2 plus TGF- β (**, *p* < 0.01; ***, *p* < 0.001).

the IL-2R β and common γ -chain, and IL-7, a cytokine that uses the IL-2R common γ -chain to substitute for IL-2. TCR-stimulated CD4⁺ cells from IL-2-deficient mice did respond robustly to both

of these cytokines with marked CD25 expression, but neither of these cytokines could enable TGF- β to induce TCR-stimulated CD4⁺ cells from these mice to express Foxp3. A similar result was obtained with IL-4 (results not shown). However, replacement of IL-2 with exogenous IL-2 restored the ability of TGF- β to induce Foxp3 (Fig. 1C). These studies suggest that IL-2 may have a non-redundant role in enabling TGF- β to induce CD4⁺ cells to express Foxp3.

Because TGF- β has positive, costimulatory effects on naive, unprimed CD4⁺ cells (41), we separated naive and experienced mouse CD4⁺CD25⁻ cells into CD62L-positive and -negative cells. IL-2 and TGF- β consistently induced more naive CD4⁺CD25⁻ cells than memory CD4⁺CD25⁻ cells to express Foxp3 (Fig. 2). In addition, IL-2 and TGF- β -induced naive CD4⁺ cells resulted in greater suppressive activity compared with memory CD4⁺ cells (data not

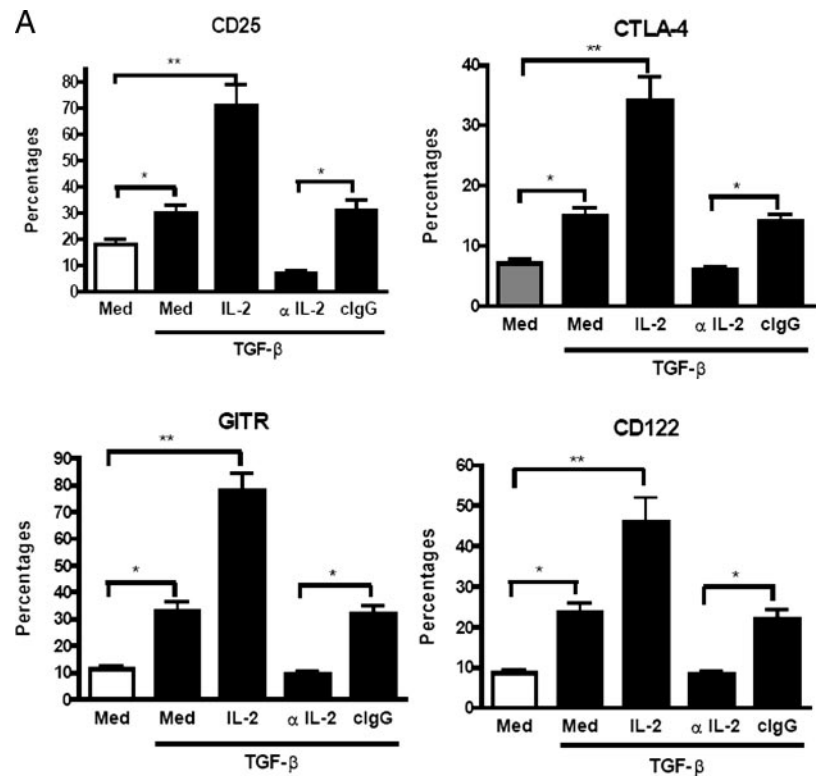


FIGURE 5. IL-2 is required for TGF- β to induce CD4⁺ cells to express a phenotype similar to natural CD4⁺CD25⁺ regulatory cells. **A**, Expression of CD25, CTLA-4, GITR, and CD122 in the TGF- β -treated CD4⁺ cells in the presence of IL-2 (20 U/ml) or anti-IL-2 (2 μ g/ml). Naive CD4⁺ cells were polyclonally stimulated \pm IL-2 \pm TGF- β for 4 days. In some experiments, anti-IL-2 or control IgG was added to cultures. Values indicate the mean \pm SEM of three separate experiments. Values of *p* were calculated by Student's *t* test (*, *p* < 0.05; **, *p* < 0.01). **B**, Coexpression of CD25, GITR, CTLA-4, or CD122 and Foxp3 in polyclonally activated CD4⁺ cells primed with IL-2 or IL-2+ TGF- β . Percentages of cells within designated quadrants are indicated. The data are representative of five independent experiments.

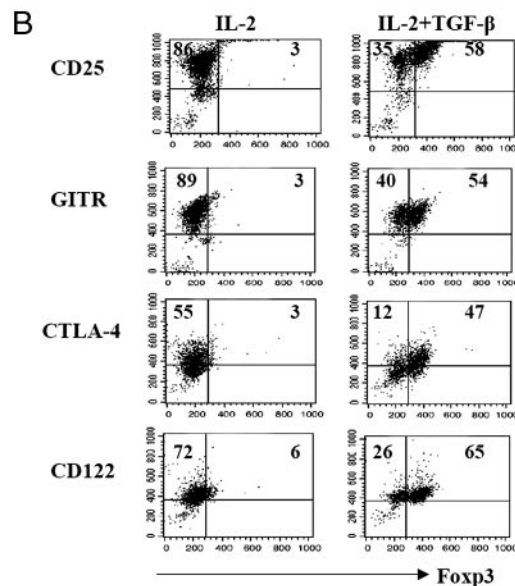
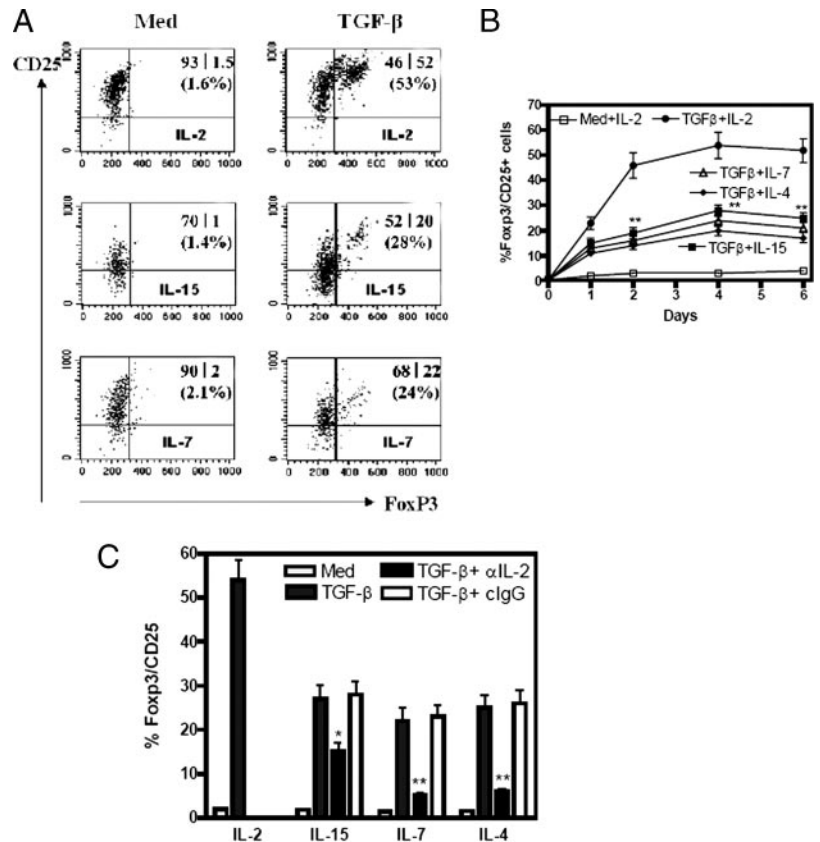


FIGURE 6. Other IL-2R β or common γ -chain cytokines cannot substitute for IL-2 in enabling TGF- β to induce Foxp3. **A**, Splenic naive CD4⁺CD25⁻ cells from C57BL/6 mice were polyclonally stimulated \pm TGF- β for 4 days with IL-7 (100 ng/ml) or IL-15 (100 ng/ml). IL-2 (20 U/ml) was used as the positive control. The number of CD25 and Foxp3-positive cells and the percentages of CD25⁺ cells expressing Foxp3 is shown in the upper right quadrant of a dot plot that is representative of five independent experiments. **B**, Time course of Foxp3 and CD25 expression using a similar experimental design as in **A**. Values indicate the mean \pm SEM of four separate experiments. Values of p indicate significant differences between TGF- β -treated cells with IL-2 and TGF- β with IL-4 (10 ng/ml), IL-7, or IL-15 at the indicated times (**, $p < 0.01$). **C**, Effects of IL-4, IL-7, and IL-15 can be partially explained by IL-2. Anti-IL-2 (2 μ g/ml) or control IgG was added to cultures containing these cytokines. Values indicate the mean \pm SEM of four independent experiments. Values of p indicate the significant difference between anti-IL-2-treated or control IgG-treated groups (*, $p < 0.05$; **, $p < 0.01$).



shown). Therefore, we used naive CD62L⁺CD4⁺CD25⁻ cells in all subsequent experiments.

Besides enabling TGF- β to induce Foxp3, IL-2 could also expand these Foxp3⁺ cells. To distinguish between these two possibilities, naive CD4⁺CD25⁻ cells were labeled with CFSE and stimulated with TGF- β . Either low-dose anti-IL-2 or exogenous IL-2 was added to some cultures. Fig. 3, **A** and **B**, shows that the starting population did not express Foxp3 protein or mRNA. Before the cells began to divide at day 1, 15–20% of CD4⁺ cells expressed Foxp3. By day 2, 30–35% of the cells expressed Foxp3. Almost all of these cells had divided at least once and reflected expansion of the starting Foxp3⁺ population. The addition of exogenous IL-2 did not immediately affect the numbers of Foxp3⁺ cells. Without exogenous IL-2, the numbers of Foxp3⁺ cells had begun to decrease by day 4. With IL-2, however, they continued to increase (Fig. 3, **C** and **D**). We also observed that, as before, anti-IL-2 abolished the ability of TGF- β to induce Foxp3, but the dose required for this effect did not block the expansion of Foxp3⁺ cells. Thus, IL-2 is not only required to enable TGF- β to induce Foxp3, but also for the expansion of these CD4⁺Foxp3⁺ cells.

Exogenous IL-2 enhances the numbers of CD4⁺Foxp3⁺ cells that develop regulatory cell phenotype and suppressive activity

In addition to endogenous IL-2 produced by CD4⁺ cells responding to TCR stimulation, small amounts of IL-2 added to the cultures markedly enhanced TGF- β -induced Foxp3 expression. The addition of 2 U/ml was just as effective as greater doses in increasing Foxp3 mRNA and protein (Fig. 4, **A** and **C**). However, raising the IL-2 dose increased the total number of CD4⁺Foxp3⁺ cells (Fig. 4**B**), a result due to expansion of these cells.

The addition of exogenous IL-2 to TGF- β not only increased numbers of Foxp3⁺ cells, but also enhanced suppressive activity. As we and others (8–11, 18–20) have shown, T cells stimulated

with TGF- β developed the ability to inhibit T cell proliferation. Although the addition of IL-2 only had no effect on the generation of suppressive activity, the combination of IL-2 and TGF- β significantly increased both the number of Foxp3⁺ cells and suppressive activity (Table I).

Where we substituted IL-15 for IL-2 in cultures with TGF- β , the CD4⁺ cells did not develop suppressive activity (results not shown). Of note, the increased inhibitory effect of TGF- β -induced CD4⁺ cells by IL-2 is closely related to the increased Foxp3 expression (Table I). To fairly compare the suppressive effect of CD4⁺ cells treated with TGF- β or TGF- β +IL-2, we further sorted CD25⁺ subsets from the CD4⁺-conditioned cells and added the purified CD25⁺ cells to the responder cells. As expected, CD25⁺ subsets from TGF- β +IL-2-treated CD4⁺ cells displayed the more potent suppressive activity than CD25⁺ subsets from CD4⁺ cells treated with TGF- β alone (Fig. 4**D**). Again, this increased suppressive capacity of CD25⁺ subsets can be explained by the increased Foxp3 expression (Fig. 4**D**).

We reported previously that TCR stimulation of CD4⁺ cells with TGF- β enhances expression of CD25, CD122, CTLA-4, and GITR (8, 16, 19, 20). Fig. 5**A** shows that up-regulation of each of these markers by TGF- β was dependent upon the presence of IL-2 because the addition of small amounts of anti-IL-2 blocked this enhancement. Although the addition of exogenous IL-2 without TGF- β to the cultures also resulted in high levels of each of these markers, the cells did not express Foxp3 (Fig. 5**B**). With the combination of IL-2 and TGF- β , however, from 60 to 80% of the cells expressing these markers also coexpressed Foxp3. CD4⁺ cells that did not exhibit any of these markers rarely expressed Foxp3. Thus, the vast majority of CD4⁺CD25⁻ cells that are activated with IL-2 and TGF- β become Foxp3⁺ cells that have the phenotype and suppressive effects similar to natural CD4⁺CD25⁺ cells.

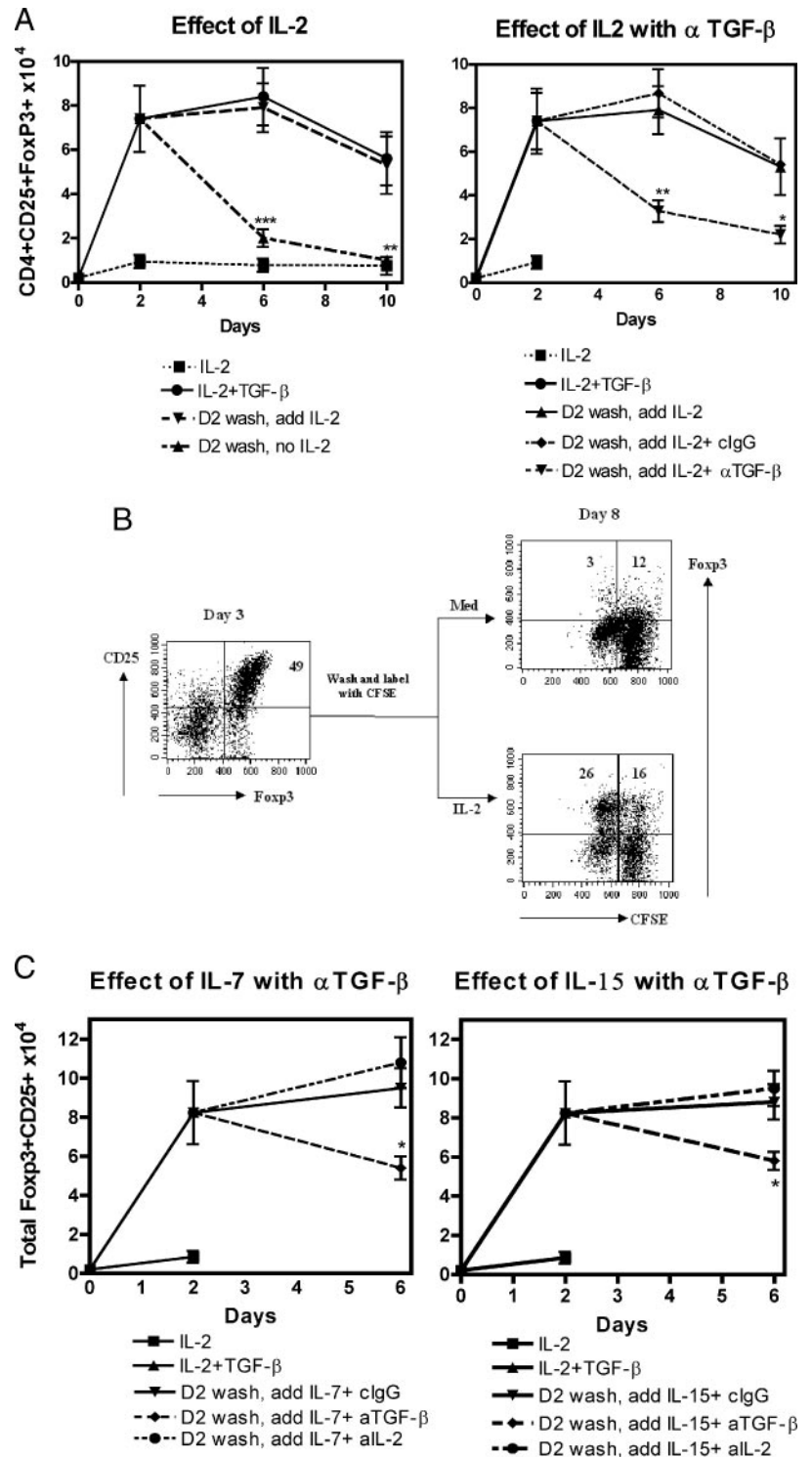


FIGURE 7. Sustained expression of TGF- β -induced Foxp3 depends upon both IL-2 and TGF- β . **A**, Naive CD4⁺CD25⁻ cells were polyclonally stimulated with anti-CD3 plus anti-CD28 beads with IL-2 (20 U/ml) \pm TGF- β for 2 days. These cells were extensively washed and recultured with medium alone, or with IL-2 (20 U/ml), IL-2 plus anti-TGF- β (10 μ g/ml), or IL-2 plus control IgG for an additional 8 days. Foxp3 and CD25 expression by CD4⁺ cells on days indicated was measured by FACS. The absolute numbers of CD4⁺CD25⁺Foxp3⁺ cells were calculated by multiplying the percentages of CD4⁺CD25⁺Foxp3⁺ cells by the total cell numbers. Values indicate the mean \pm SEM of three separate experiments. Values of p indicate a significant decrease in expression of Foxp3 following the removal of IL-2 on the days indicated (*left panel*: **, $p < 0.01$; ***, $p < 0.001$) or the addition of anti-TGF- β (*right panel*: *, $p < 0.05$; **, $p < 0.01$). **B**, Similar experiments as described in **A**. After 3 days, the cells were washed and coated beads were removed. The cells were then labeled with CFSE and recultured with or without IL-2 (20 u/ml) for an additional 5 days. Foxp3, CD25, and CFSE expression by CD4⁺ cells on days indicated was measured by FACS. The results are representative of three independent experiments. **C**, Similar experiments as described in **A**. After 2 days, the cells were recultured with IL-7 (100 ng/ml), IL-15 (100 ng/ml), and anti-TGF- β (10 μ g/ml) or control IgG for an additional 4 days. Values indicate the mean \pm SEM of three separate experiments. Values of p indicate significant differences between cultures with anti-TGF- β and control IgG (*, $p < 0.05$).

As described above, the effect of IL-2 on enhancing TGF- β -induced Foxp3 was largely nonredundant. Substituting IL-7 or IL-15 for IL-2 resulted in much less TGF- β -induced Foxp3. As shown in Fig. 6, **A** and **B**, the addition of each of these cytokines to TCR-stimulated CD4⁺ cells markedly increased CD25 expression. However, Foxp3 increased only when TGF- β was added. Most of this increase, however, was due to the IL-2 produced by the activated T cells, because the addition of anti-IL-2 to cultures with IL-4, IL-7, and IL-15 markedly decreased Foxp3 expression (Fig. 6C). In addition, these common γ -chain cytokines could not replace IL-2 in generating the expansion of the Foxp3⁺ population

(Fig. 6B). These data suggest that IL-2 exclusively enhances the Foxp3 expression and suppressive activity on TGF- β -induced CD4⁺CD25⁺ cells.

Sustained expression of TGF- β -induced Foxp3 depends upon both IL-2 and TGF- β

We next determined whether the combination of IL-2 and TGF- β was also needed for sustained expression of Foxp3. In the experiment shown in Fig. 7A where naive CD4⁺ cells had received TCR stimulation with IL-2 and TGF- β , by 48 h 46% of those that exhibited CD25 had become Foxp3⁺. If the cytokines were removed

at this time Foxp3 levels rapidly decreased. The addition of IL-2, however, maintained and CFSE-labeling studies demonstrated expansion of the Foxp3⁺ cells (Fig. 7B). As described before, naive CD4⁺ cells activated with IL-2 only did not express Foxp3.

Furthermore, the presence of TGF- β produced by the TCR-stimulated cells was also necessary for sustained expression of Foxp3. We observed that the addition of anti-TGF- β to IL-2 resulted in the same decay of this transcription factor as in cultures lacking IL-2 (Fig. 7A). Another series of experiments performed after 6 days of TCR stimulation with TGF- β showed similar dependence on IL-2 to sustain Foxp3 expression (results not shown). Thus, CD4⁺ cells require continuous exposure to both IL-2 and TGF- β for sustained expression of Foxp3.

Unlike the induction and expansion of Foxp3, the effect of IL-2 on sustaining Foxp3 was not nonredundant. Once the CD4⁺ cells expressed Foxp3, the presence of IL-7 or IL-15 could sustain the expression of this transcription factor. However, the effect of these cytokines also partially depended upon the presence of TGF- β produced by the activated CD4⁺ cells because neutralization of this cytokine resulted in a decay of Foxp3 expression (Fig. 7C).

Discussion

Both IL-2 and TGF- β have separately been reported to have important roles in the development, survival, and function of CD4⁺CD25⁺ Treg cells (7–11, 18–21, 34–38). This study documents that these two cytokines act in concert to induce naive CD4⁺CD25⁻ cells to become CD25⁺Foxp3⁺ Tregs, and the two together also sustain them. By themselves, neither of these cytokines had this property. Additionally, IL-2 also plays a unique role in the expansion of Foxp3⁺ suppressor cells. We have previously documented that TGF- β requires TCR stimulation by polyclonal mitogens, superantigens, or alloantigens to induce CD4⁺ or CD8⁺ Tregs (8, 16, 18–20, 39). Subsequently, it has become evident that TGF- β induces naive CD4⁺CD25⁻ cells to express Foxp3, the transcription factor responsible for the development of natural CD4⁺CD25⁺ cells (4, 5, 7–10). In this study, we have used Ab neutralization studies and experiments with IL-2-deficient mice to delineate the effects of IL-2 on the regulatory properties of TGF- β . Our observations suggest that the role of IL-2 in enabling TGF- β to induce CD4reg is nonredundant. Because several groups have recently reported that IL-2 is nonessential for the development of Foxp3⁺ natural Treg cells (26, 30, 35–37), the synergistic effects of IL-2 and TGF- β on Foxp3 expression described in this study distinguishes the generation of natural Treg from that of TGF- β -induced, adaptive Treg cells.

Although agreement exists that IL-2 has an important role in the maintenance of tolerance to self Ags, whether these effects are direct or indirect has been poorly understood. Recently, another group has reported a role for IL-2 in the generation of peripheral CD4⁺CD25⁺ Treg cells in vivo (42), but the mechanism of action of IL-2 was not addressed. Although Zorn et al. (43) reported that IL-2 selectively up-regulates the expression of Foxp3 in purified CD4⁺CD25⁺ T cells but not in CD4⁺CD25⁻ cells, the results of the present experiments suggest that the tolerizing effects of IL-2 on peripheral T cells are indirect. To address this issue, we avoided mixing APCs with CD4⁺ cells to exclude the possible confounding effects that the costimulatory molecules and cytokines produced by these cells would contribute to the regulatory effects of TGF- β . We observed that blocking signaling of endogenously produced IL-2 abolished the ability of TGF- β to induce CD4⁺ cells to express Foxp3 and become suppressor cells. Secondly, TGF- β was unable to induce CD4⁺ cells from IL-2^{-/-} mice to become Foxp3⁺ Treg cells. Moreover, although cytokines such as IL-15 and IL-7 that bind to the common γ -chain and/or IL-2R β -chain

were able to markedly augment CD4⁺ cell activation in IL-2-deficient mice, these cytokines could not substitute for IL-2 in enabling TGF- β to induce Foxp3. IL-7 and IL-15 have been reported to abolish the suppressive activity of Treg cells (44). Thus, the positive effect of IL-2 on TGF- β -induced CD4reg is probably nonredundant.

Others (41) have reported that TGF- β has preferential costimulatory effects on naive T cells. In this study, we observed that TGF- β induced a greater percentage of naive CD4⁺CD62L⁺CD25⁻ cells to express Foxp3 than experienced CD4⁺CD62L⁻CD25⁻ cells, possibly because naive T cells produce predominantly IL-2. Time course studies revealed that IL-2 had two distinct effects. This cytokine was needed for TGF- β to induce CD4⁺CD25⁻ cells to express Foxp3, and also required for the expansion of these cells. Before the cells began to divide 15–20% of naive CD4⁺CD25⁻ stimulated with both cytokines became Foxp3⁺, and the subsequent CD4⁺ cells expressing this transcription factor were derived from proliferating cells.

There were several reasons why the addition of exogenous IL-2 to the cultures increased suppressive activity: 1) this cytokine increased the percentage of Foxp3⁺ cells; 2) IL-2 increased the intensity of Foxp3 expression; and 3) IL-2 expanded these cells. Although low-dose IL-2 could maximally increase the percentage of these cells, larger doses resulted in expansion. Our studies were also consistent with those who reported that IL-2 enhances the functional properties of natural CD4⁺CD25⁺ Treg cells (34, 35). With the latter, IL-2 was provided by CD4⁺CD25⁻ cells because natural CD4reg cells do not produce this cytokine (1, 2). As described above, enhancement of Foxp3 expression by exogenous IL-2 also appeared to be nonredundant. The induction of modest amounts of Foxp3 observed with exogenous IL-7 or IL-15 could be explained by enhanced T cell activation and secondarily produced IL-2.

Synergistic effects of IL-2 and TGF- β were also needed for naive CD4⁺CD25⁻ cells to develop the phenotype of CD4⁺CD25⁺ Treg cells. As reported by others (45), TGF- β enhances CD25 expression by TCR-stimulated CD4⁺ cells and partial neutralization of endogenous IL-2 blocked this effect. TGF- β -induced Treg cells also exhibit CD122, GITR, and CTLA-4, all markers expressed by activated CD4⁺ cells. We and others (8–11, 19, 20) have previously reported that TGF- β enhances the ability of TCR-stimulated CD4⁺CD25⁻ cells to express these markers. Although IL-2 by itself can increase the expression of these markers, few of these cells express Foxp3. The combination of IL-2 and TGF- β was necessary for coexpression of these markers with Foxp3 (Fig. 5).

Not only does IL-2 and TGF- β induce Foxp3 expression, but both cytokines were needed to sustain expression of this transcription factor. Other workers have reported that IL-2 is necessary for the survival and “fitness” of natural Treg cells (26, 36, 37). In these experiments, expression of Foxp3 by CD4⁺ cells stimulated with IL-2 and TGF- β was transient. Levels fell rapidly when these two cytokines were removed from the cultures. Although the addition of IL-2 by itself could sustain Foxp3 expression, Ab neutralization experiments suggested that T cell-derived TGF- β was also needed for this effect. TGF- β has paracrine effects that enable T cells to up-regulate production of this cytokine (46, 47), and the presence of TGF- β at the onset of the cultures induced production of this cytokine. Moreover, it is now appreciated that T cells not only release cytokines into the microenvironment, but also secrete them into immunological synapses where they can modulate TCR signaling (48). This contact-dependent cytokine effect may explain why anti-TGF- β blocks the generation of secondarily induced Treg cells before secretion of this cytokine became detectable (49).

The cross-talk between IL-2 and TGF- β may have other positive effects. TGF- β can protect IL-2-activated T cells from apoptosis (41, 49), and TGF- β enhances IL-2-mediated expression of CD25 (45).

Our observation that TGF- β is required to maintain Foxp3 expression is consistent with the recent report that TGF- β 1 signaling is required for CD4⁺CD25⁺ Treg cells to sustain Foxp3 expression and suppressor cell function (21). T cells produce TGF- β as a latent precursor that needs to be converted into a biologically active form (46, 47, 50). Activated T cells express the urokinase plasminogen activator receptor that converts latent to active TGF- β 1 and IL-2 up-regulates this activity (51, 52). Thus, one function of IL-2 could be to support the constant source of active TGF- β that is needed to maintain Treg Foxp3 expression.

The signaling pathways responsible for IL-2 and TGF- β cross-talk remain to be elucidated. TGF- β , however, can inhibit the positive effects of IL-2 on T cell proliferation by uncoupling the proliferative and antiapoptotic functions of IL-2 via Smad 3 signaling (53). T cells require subimmunogenic concentrations of peptide Ag or suboptimal costimulation for induction of Foxp3 (54). The inhibitory effects of TGF- β on T cell proliferation, therefore, promote Treg rather than T effector cell differentiation. It is well known that TGF- β inhibits Th1 and Th2 differentiation (17).

In summary, evidence has been presented that the synergistic effects of IL-2 and TGF- β can induce naive CD4⁺ cells to become CD25⁺Foxp3⁺ suppressor cells that express the characteristic markers of natural Treg cells. Although IL-2 and TGF- β was required for the induction of Foxp3 by these adaptive Treg, IL-2 by itself can enhance Foxp3 expression by natural Treg and is dispensable for their development (26, 30, 35, 36, 43). The synergistic effects of IL-2 and TGF- β were also needed for sustained expression of Foxp3 by TGF- β -induced CD4reg cells. Others have also reported that TGF- β signaling is required for persistent expression of Foxp3 by natural CD4⁺CD25⁺ Treg cells. Thus, the combined effects of both natural and adaptive CD4⁺CD25⁺ Treg cells have a major role in maintaining immune tolerance and T cell homeostasis.

The observations concerning IL-2 and TGF- β described in this report have direct relevance to the pathogenesis and treatment of autoimmune diseases. In human and mouse systemic lupus erythematosus (SLE), a disorder of generalized autoimmunity, production of both IL-2 and TGF- β is decreased (55–58). Gene transfer of these cytokines ameliorated mouse lupus (59), and brief treatment of human T cells with IL-2 and TGF- β in vitro abolished autoantibody production by subjects with active SLE (60). Recent reports suggest decreased CD4⁺CD25⁺ Treg in patients with active disease (61–63). Therefore, transfer of autologous Treg cells generated ex vivo with these cytokines may have considerable therapeutic benefit.

Note added in proof. Since this paper has been accepted for publication, we have observed that IL-2 induces natural mouse and human CD4⁺CD25⁺ Treg cells to produce and express mature TGF- β on its cell surface. Similarly, natural-like Treg cells generated with TGF- β that are restimulated with IL-2 induces these CD4⁺CD25⁺ cells to also display membrane-bound TGF- β . The details of these findings will be reported separately.

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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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