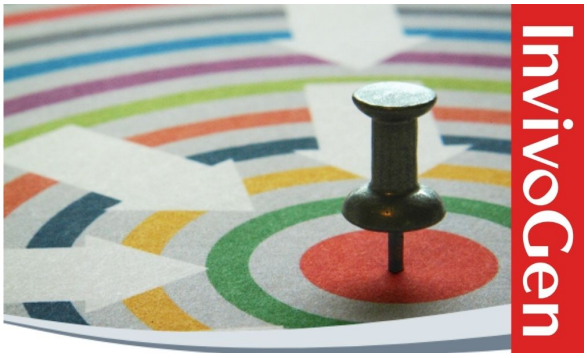

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Natural and Induced $CD4^+CD25^+$ Cells Educate $CD4^+CD25^-$ Cells to Develop Suppressive Activity: The Role of IL-2, TGF-β, and IL-10[1]

Song Guo Zheng, Ju Hua Wang, J. Dixon Gray, Harold Soucier, and David A. Horwitz[2]

Thymus-derived, natural $CD4^+CD25^+$ regulatory T cells can educate peripheral $CD4^+CD25^-$ cells to develop suppressive activity by poorly understood mechanisms. TGF-β has IL-2-dependent costimulatory effects on alloactivated naive, human $CD4^+$ T cells and induces them ex vivo to become potent contact-dependent, cytokine-independent suppressor cells. In this study, we report that $CD4^+CD25^+$ cells are the targets of the costimulatory effects of IL-2 and TGF-β. These cells do not divide, but, instead, greatly increase the numbers of $CD4^+CD25^-$ cells that become $CD25^+$ cytokine-independent suppressor cells. These $CD4^+CD25^+$ regulatory cells, in turn, induce other alloactivated $CD4^+CD25^-$ cells to become potent suppressor cells by mechanisms that, surprisingly, require both cell contact and TGF-β and IL-10. The suppressive effects of these secondary $CD4^+CD25^+$ cells depend upon TGF-β and IL-10. Moreover, both the naive $CD4^+$ cells induced by IL-2 and TGF-β to become suppressor cells, and the subsequent $CD4^+CD25^-$ cells educated by them to become suppressors express *FoxP3*. We suggest that the long-term effects of adoptively transferred natural-like $CD4^+CD25^+$ regulatory cells induced ex vivo are due to their ability to generate new cytokine-producing $CD4^+$ regulatory T cells in vivo. ***The Journal of Immunology,*** **2004, 172: 5213–5221.**

Treg (T_{reg})[3] cells are heterogeneous T cell populations that prevent harmful immune responses to self and nonself Ags. Formerly called suppressor T cells, they can be classified into two major categories: thymus-derived natural T_{reg} cells and those induced in the periphery. The principal subset of natural regulatory cells are $CD4^+$ cells that constitutively express CD25, the α-chain of the IL-2R (1–3). These have a contact-dependent, cytokine-independent mechanism of action. Peripheral T cells induced with IL-10, immature dendritic cells, or TGF-β have a cytokine-dependent mechanism of action (4–9). We have induced both $CD8^+$ and $CD4^+$ T cells to become T_{reg} cells with the combination of IL-2 and TGF-β. Depending upon the starting subset and the mode of activation, $CD4^+$ cells could become natural-like T_{reg} cells or cytokine-dependent T_{reg} cells (6–9).

In addition to their inhibitory effects, certain T_{reg} cells have the ability to educate other T cells to become suppressor cells by a phenomenon that has been called infectious tolerance. This phenomenon was first described by investigators who found that short-term treatment of rats with mAbs resulted in long-term transplantation survival. They showed that the transfer of T cells from the tolerant rats into naive rats induced a similar tolerant state (10). Further studies from this group demonstrated that $CD4^+$ T_{reg} cells played a critical role in maintaining this transplantation tolerance. Thus, these $CD4^+$ T_{reg} cells not only had suppressive function, but also educated naive T cells to become suppressor cells (11).

Recently, two groups also found that naturally occurring $CD4^+CD25^+$ T_{reg} cells directly induced conventional $CD4^+$ cells to become suppressive cells by infectious tolerance. In these studies, naturally occurring $CD4^+CD25^+$ T_{reg} cells suppressed T cell responses via a cell-contact mechanism, but the newly induced $CD4^+$ suppressor cells inhibited proliferation of $CD4^+$ T cells either via IL-10 (12) or TGF-β production (13).

We have asked whether $CD4^+CD25^+$ T_{reg} cells generated ex vivo can also educate $CD4^+CD25^-$ cells to become suppressor cells. Previously, we had reported that naive $CD4^+$ cells primed with alloantigens and TGF-β developed potent suppressive effects in ratios $<$1:100 responder T cells. Depletion of the rare ($\sim$1%) $CD4^+CD25^+$ T_{reg} cells in this fraction greatly decreased the generation of suppressive activity (8). Although the T_{reg} activity could be explained by TGF-β greatly expanding $CD4^+CD25^+$ precursor cells, it was more likely that these T_{reg} cells were interacting with $CD4^+CD25^-$ cells. In this study, we provide evidence for this hypothesis. In addition, we document that the TGF-β-induced $CD4^+CD25^+$ T_{reg} cells have the ability to educate other $CD4^+CD25^-$ cells to become cytokine-dependent T_{reg} cells and have found that TGF-β and IL-10 play pivotal roles in this phenomenon. Finally, we report that natural-like $CD4^+CD25^+$ cells induced by IL-2 and TGF-β and the educated $CD4^+$ cytokine-producing suppressor cells express *FoxP3*, the critical transcription factor required for the development of $CD4^+$ T_{reg} cells. Thus, we suggest that IL-2 and TGF-β trigger a cytokine-dependent self-perpetuating loop to sustain specific T_{reg} cell activity.

Materials and Methods

Medium

Aim V serum-free medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (all from Invitrogen, Carlsbad, CA) was used for generation of $CD4^+$ T_{reg} or control cells. RPMI 1640 medium supplemented as above with 10% heat-inactivated FCS (HyClone Laboratories, South Logan, UT) was used for all other cultures.

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[3] Abbreviations used in this paper: T_{reg}, T regulatory; GITR, glucocorticoid-induced TNF family related receptor.

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Abs and cytokines

The following Abs were used: anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CTLA-4, anti-CD122, anti-CD62L, anti-HLA-A2, and respective mouse and rat isotype controls (all from BD PharMingen, San Diego, CA); anti-glucocorticoid-induced TNF family related receptor (GITR) and control IgG (R&D Systems, Minneapolis, MN); anti-CD8, anti-CD11b, and anti-CD74 (all hybridomas from American Type Culture Collection, Manassas, VA); and anti-CD16 (3G8; kindly provided by J. Unkeless, Mount Sinai Medical School, New York, NY). Abs used for intracellular cytokine staining were FITC- or PE-conjugated anti-IL-2, anti-IL-10, anti-IFN-γ, and anti-TNF-α (all from eBioscience, San Diego, CA). Unconjugated anti-IL-2, anti-IL-10, anti-TGF-β, and matched control IgG Abs (all from R&D Systems) were used for the neutralization experiments; anti-CD3 and anti-CD28 beads (the gift from C. June, University of Pennsylvania, Philadelphia, PA) were used for polyclonal activation of T cells. Recombinant human IL-2 and TGF-β1 were purchased from R&D Systems.

Lymphocyte isolation

T cells and T-depleted (stimulator) cells were prepared from heparinized venous blood of healthy adult volunteers, as described previously (9). CD4$^+$ or naive CD4$^+$ cells were prepared from T cells that were stained with Abs to CD8 or CD8 and anti-UCHL-1 (CD45RO), then isolated by negative selection using immunomagnetic beads (Dynal Biotech, Great Neck, NY). CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were isolated from these populations by cell sorting gating on CD25 bright cells using a FACSDiVa (BD Biosciences, San Jose, CA). In some experiments, the CD25$^+$ and CD25$^-$ subsets were isolated by CD25 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with >95% purity.

Generation of CD4$^+$ T_{reg} cell subsets

We have used three sequential cell cultures to determine the role of the few CD25$^+$ cells in the naive fraction of CD4$^+$ cells in TGF-β-induced T_{reg} activity, and to document effects of these T_{reg} cells on the induction of new T_{reg} cells from CD25$^-$ precursors. In primary cultures, purified naive CD4$^+$ cells were added to irradiated (30 Gy) allogeneic stimulator cells for 6 days ±TGF-β (0.1–10 ng/ml). In some experiments, rIL-2 (10 U/ml) was also added to the cultures. The CD4$^+$CD25$^+$ cells were then isolated by cell sorting. Those primed with TGF-β are called T_{reg}1, and those activated without this cytokine are called T_{con}1.

To track the response of CD4$^+$CD25$^+$ cells to alloantigens, we used donors who differed in HLA-A2 expression. Naive CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells (2×10^5) from one donor and 2×10^6 CD4$^+$CD25$^-$ cells from another donor were activated with third party allogeneic stimulator cells. After 6 days of coculture, the CD4$^+$ cells were stained with Abs to CD25, CD122, GITR, or isotype controls and analyzed by flow cytometry.

To determine whether T_{reg}1 could induce other CD4$^+$CD25$^-$ cells to become suppressor cell, they were added to a fresh allostimulatory culture. To distinguish between the two CD4 populations, the CD4$^+$CD25$^-$ cells were labeled with CFSE (Molecular Probes, Eugene, OR). The labeled CD4$^+$CD25$^-$ cells (2×10^6) were activated with equivalent numbers of irradiated allogeneic stimulator cells for 6 days. In some cultures, $2–4 \times 10^5$ T_{reg}1 or T_{con}1 were added to these cultures. Six days later, three distinct cell populations could be identified by flow cytometry. The undivided cells stained brightly for CFSE (CD4$^+$CFSE^{high}), divided cells had diluted the CFSE (CD4$^+$CFSE^{mod}), and primed CD4$^+$CD25$^+$ cells were unlabeled (CD4$^+$CFSE$^-$). The different populations were isolated by cell sorting. We obtained CD4$^+$CFSE$^-$ cells (T_{reg}1 or T_{con}1), CFSE moderately positive cells (T_{reg}2 or T_{con}2), or nondivided, CFSE bright cells (T_{reg}3 or T_{con}3).

Cytokine assays

Primed CD4$^+$ cells were extensively washed and restimulated with either allogenic stimulators or anti-CD3/28 beads (1:20) for 24, 48, 72, 96, and 120 h in serum-free AIM V medium for TGF-β production, and with complete medium for production of other cytokines. Active TGF-β was determined by mink lung epithelial cells transfected with a luciferase gene construct (14). Supernatants were also tested in duplicate using ELISA kits (BioSource International, Camarillo, CA) for IL-10 and IFN-γ, according to the manufacturer's instructions. For analysis of intracellular cytokine production, CD4$^+$ cells were stimulated with 100 ng/ml PMA and 5 μM

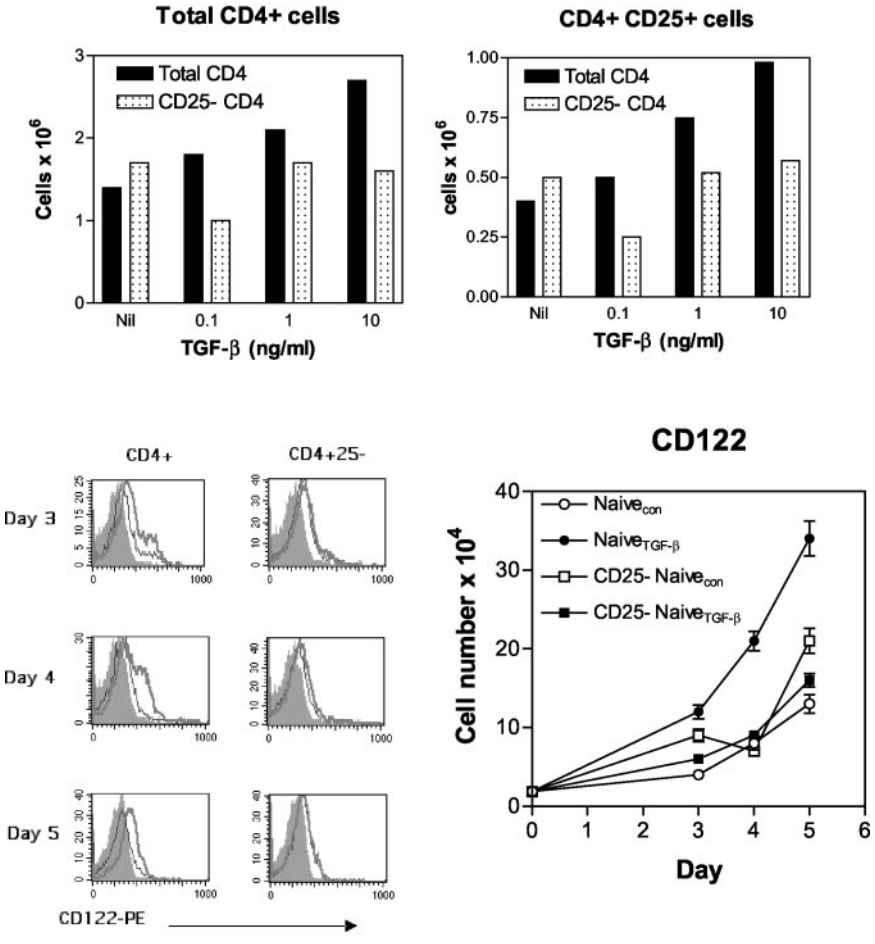

FIGURE 1. Costimulatory effects of IL-2 and TGF-β on alloactivated naive CD4$^+$ T cells. The costimulatory effects are dependent upon CD4$^+$CD25$^+$ T cells. One million CD4$^+$CD45RO$^-$ cells or naive CD4$^+$ cells depleted of CD25$^+$ cells from donor A were mixed with equal numbers of irradiated non-T cells from donor B with IL-2 (10 U/ml) ± TGF-β1 in the doses shown. Two separate experiments are shown. *Top*, The cells were incubated for 6 days, counted, stained for CD25, and examined by flow cytometry (CD25 mean fluorescence intensity, total CD4$^+$ cells ± TGF-β (10 ng/ml) 405 vs 578; CD25$^-$CD4$^+$485 vs 511). *Bottom*, Samples of the cells were harvested at 3, 4, and 5 days, counted in triplicate, and stained for CD122. Depletion of CD25$^+$ cells completely abolished the TGF-β-mediated enhancement of IL-2R α- and β-chains. The experiments shown are representative of at least eight experiments examining CD25 and CD122.

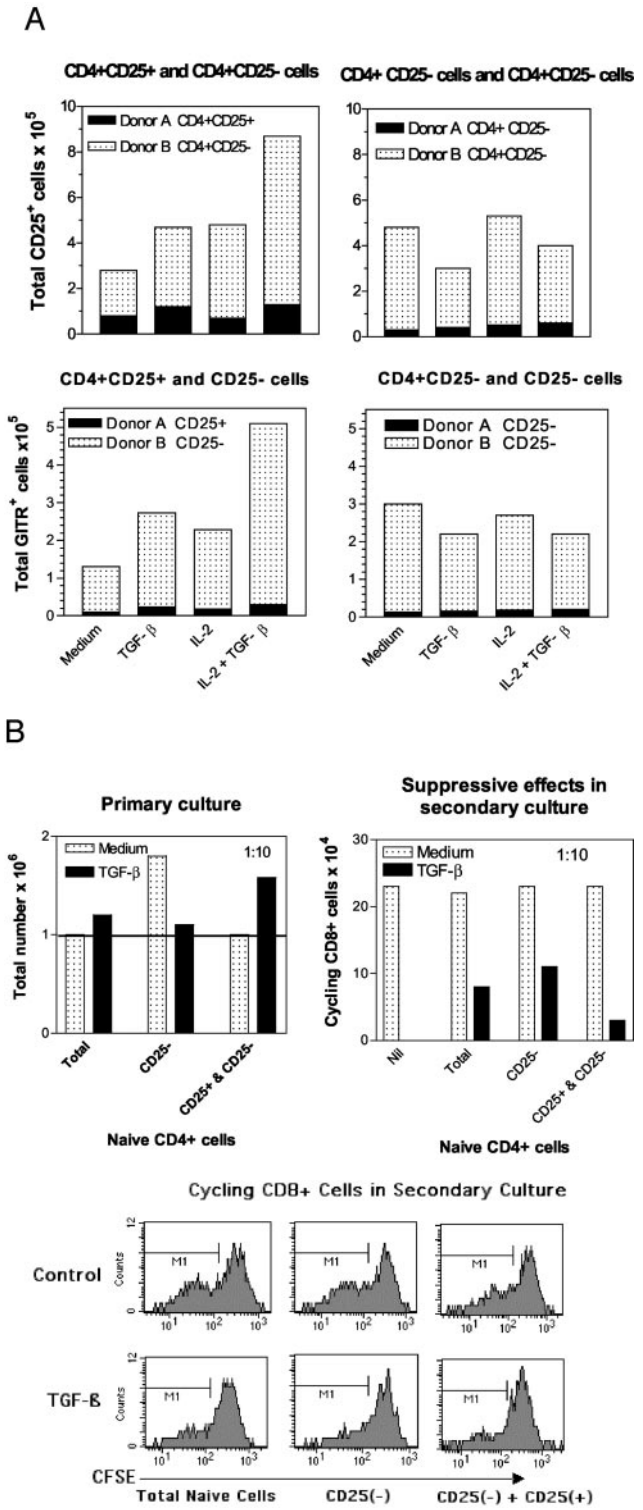

FIGURE 2. TGF-β-stimulated $CD4^+CD25^+$ cells expand $CD4^+CD25^-$ cells and augment their suppressive activity. *A*, IL-2 and TGF-β alloactivated $CD4^+CD25^+$ cells induce $CD4^+CD25^-$ cells to expand and express markers characteristic of T_{reg} cells. Naive $CD4^+CD25^+$ cells or control $CD4^+CD25^-$ were prepared from donor A, who was HLA-A2 negative, and $CD4^+CD25^-$ cells were prepared from donor B, who was $HLA\text{-}A2^+$. One million $CD4^+CD25^-$ cells from donor B were mixed with $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from donor A in a 1:10 ratio, and cultured with one million irradiated non-T cells from donor C. Some wells contained medium only, TGF-β1 (10 ng/ml), IL-2 (10 U/ml), or both TGF-β and IL-2. The cells were harvested after 6 days, counted, and triple stained for CD4, HLA-A2, and CD25 or GITR. Gating on $CD4^+$ cells, the $CD25^+$ and $GITR^+$ cells from each donor could be counted and the solute numbers determined. This experiment was repeated twice with similar results. *B*, Naive $CD4^+$ cells from one donor were separated into $CD25^+$ and $CD25^-$ cells. One million total naive $CD4^+$ cells, CD25-depleted cells, or $CD25^+$ and $CD25^-$ cells mixed together in a 1:10 ratio were cultured with irradiated stimulator cells ± TGF-β1 (10 ng/ml) without additional IL-2 in this experiment. After 6 days, the cells were harvested, each well was counted, and each subset was mixed with CFSE-labeled autologous T cells in a 1:10 ratio. The cells were restimulated for 6 days, counted, and stained for CD8, and the number of $CD8^+$ cells that had divided was determined by flow cytometry. The panels show the numbers of each subset in cultures with and without TGF-β. *Left*, Changes in $CD4^+$ cells in cultures with and without TGF-β. The horizontal line denotes the number at baseline. *Right*, Suppressive effects of naive $CD4^+$ cells primed with TGF-β on dividing, CFSE-labeled $CD8^+$ cells. TGF-β- and control-primed $CD4^+$ cells were mixed with CFSE-labeled T cells in a 1:10 ratio. *Bottom*, Histogram of gated $CD8^+$ cells showing the inhibitory effects of $CD4^+$ cells primed with TGF-β. One of six similar experiments is shown.

ionomycin for the last 6 h of culture. Brefeldin A (10 μg/ml) was added for last 5 h of culture. Cells were harvested, then fixed and permeabilized (Fix and Perm; Caltag Laboratories, Burlingame, CA), and stained with cytokine-specific Abs or isotype controls.

Assays for suppressive activity

To determine the suppressive activity of the various T_{reg} subsets, we measured their ability to inhibit the generation of autologous alloreactive $CD8^+$ cells in a CTL cytotoxicity assay, as previously described (8). The ability of these cells to inhibit the $CD8^+$ cell activation and proliferation induced by allogeneic stimulator cells was determined by measuring CD25 expression or cycling of CFSE-labeled $CD8^+$ cells.

FoxP3 expression by real-time RT-PCR

Total RNA was prepared with TRIzol LS reagent (Invitrogen). First strand cDNA was synthesized using Omniscript TR kit (Qiagen, Valencia, CA) with random hexamer primers (Invitrogen). Real-time PCR was performed with a LightCycler (Roche, Mannheim, Germany), and message levels were quantified using the LightCycler Fast Start DNA Master SYBR Green I Kit (Roche), according to the manufacturer's instructions. Amplification was conducted for 45 cycles. The recovered PCR product and amplicon were checked by agarose gel electrophoresis for a single band of the expected size. The relative expression of FoxP3 was determined by normalizing expression of each target to GAPDH. Primer sequences were as follows: GAPDH, 5′-CCACATCGCTCAGACACCAT-3′ and 5′-GGCA ACAATATCCACTTTACCAGAGT-3′; FoxP3, 5′-GAAACAGCACAT TCCCAGAGTTC-3′ and 5′-ATGGCCCAGCGGATGAG-3′.

Results and Discussion

Costimulatory effects of TGF-β on naive $CD4^+CD25^+$ cells

We have previously reported that TGF-β has costimulatory effects on alloactivated naive $CD4^+$ T cells, as indicated by up-regulation of CD25 and CTLA-4 and an expansion of these cells (8). These costimulatory effects were dependent upon a sufficient amount of IL-2 to overcome the inhibitory effects of TGF-β. In this study, we report that a target of these costimulatory effects is the $CD4^+CD25^+$ cell subset, even though they constitute only 1% of naive $CD4^+$ cells. Fig. 1 shows that a dose-dependent increase in CD25 expression was completely abolished by depletion of $CD25^+$ cells in the starting population. TGF-β also significantly enhanced expression of CD122, the β-chain of the IL-2R. Signaling through CD122 is essential for the suppressive activity of $CD4^+CD25^+$ cells (15,16). TGF-β-mediated enhancement of CD122 expression was apparent at day 3 and was maximal 1 day later. The few $CD25^+$ cells present in the naive fraction resulted in a 4-fold expansion of CD122-bearing cells. Like CD25 expression, depletion of the $CD25^+$ cells abolished this enhancement.

The next step was to learn whether TGF-β increased the expansion and potency of the $CD25^+$ subset, or whether the $CD25^+$ cells acted on $CD25^-$ cells. Pilot experiments on allostimulated purified $CD4^+CD25^+$ cells revealed that expansion of these cells

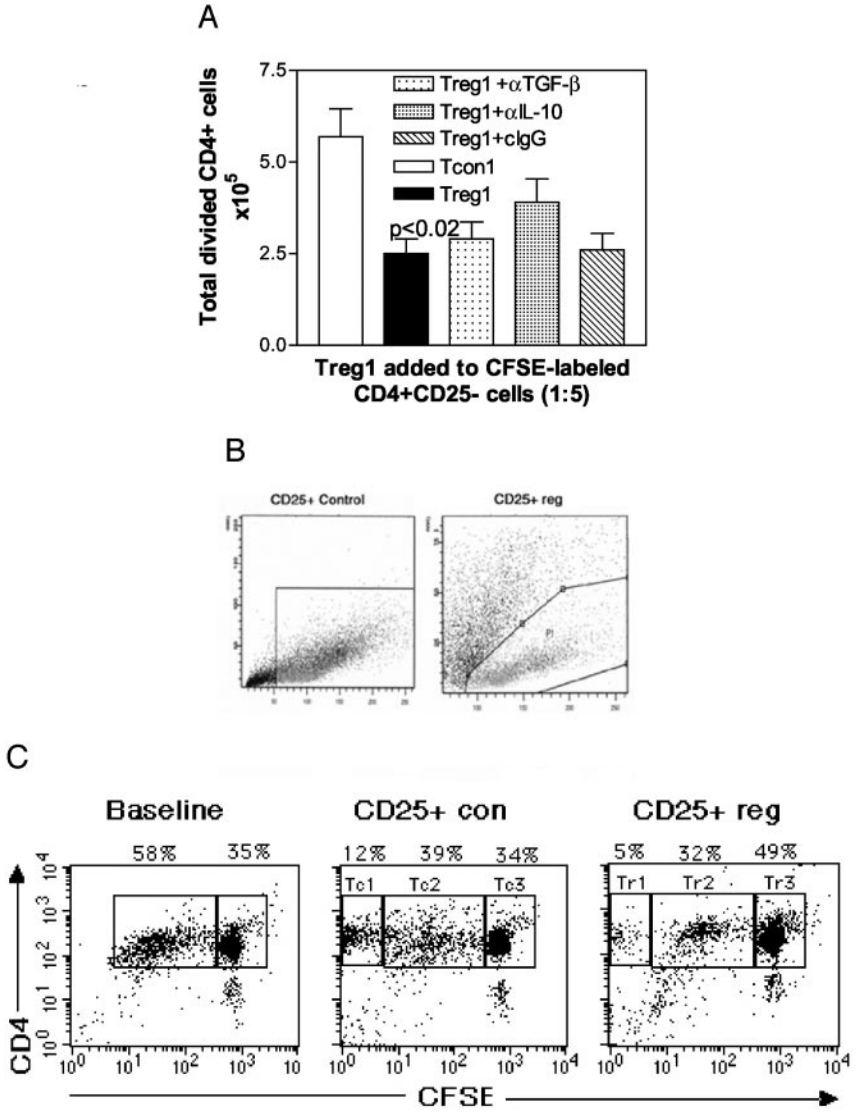

FIGURE 3. $T_{reg}1$ inhibit most allostimulated $CD4^+CD25^-$ cells, but allow a subset to proliferate. *A*, Cytokine-independent inhibitory effects of $T_{reg}1$. A total of 4×10^5 $CD4^+$ cells, T_{reg} 1 cells, or T_{con} 1 cells and 2×10^6 CFSE-labeled $CD4^+CD25^-$ cells from donor A was stimulated with 2×10^6 irradiated non-T cells from donor B for 6 days. Some wells contained anti-TGF-β, anti-IL-10, or control IgG. The mean of 10 separate experiments ± SEM shows that $T_{reg}1$ decreased the numbers of proliferating $CD4^+$ cells in comparison with $T_{con}1$. *B*, Flow cytometry scatter profile of a representative experiment showing the number of viable cells. $T_{reg}1$ cells resulted in markedly fewer viable cells. *C*, The cells were stained for CD4 and analyzed by flow cytometry. Wells that contained CFSE-labeled $CD4^+CD25^-$ responder cells without primed $CD4^+$ cells are shown along with wells containing $T_{con}1$ cells or $T_{reg}1$ cells. CFSE-labeled $CD4^+$ cells that had divided are called $T_{con}2$ cells or $T_{reg}2$ cells, and those that had not divided are called $T_{con}3$ cells or $T_{reg}3$ cells. After pilot studies revealed that $T_{con}3$ and $T_{reg}3$ did not have inhibitory effects, they were not studied further.

was only minimal, and that the combination of IL-2 and TGF-β did not substantially increase their suppressive activity (results not shown). To distinguish the progeny of $CD4^+CD25^+$ cells and $CD4^+CD25^-$ cells, we used cells from donors that differed in HLA-A2 expression. These studies conclusively demonstrated that $CD25^+$ cells costimulated the $CD25^-$ cells. Naive $CD4^+CD25^+$ or $CD25^-$ cells from one donor were added to $CD25^-$ cells from another donor in a 1:10 ratio, and these cells were stimulated by irradiated non-T cells from a third donor. The presence of $CD25^+$ cells enabled IL-2 and TGF-β to increase the number of $CD25^+$ cells by 2-fold, and $GITR^+$ cells by 5-fold (Fig. 2*A*). This expansion, however, was derived predominantly from $CD25^-$ precursors. Depletion of the $CD25^+$ T_{reg} cells resulted in a more vigorous MLR response, but this effect was not modified by the addition of IL-2 or TGF-β.

The experiment shown in Fig. 2*B* shows that naive $CD4^+$ $CD25^+$ cells not only enable TGF-β to increase the cell number of $CD4^+CD25^-$ cells, but probably also augment their suppressive activity. In a primary MLR, 10% $CD4^+CD25^+$ cells resulted in a 60% increase in cell numbers in the presence of TGF-β. Although TGF-β could induce CD25-depleted $CD4^+$ cells to suppress the proliferative response of $CD8^+$ cells to alloantigens, this activity was markedly enhanced if the starting population also contained $CD25^+$ cells. Suppression of both the percentage and total numbers of dividing $CD8^+$ cells was increased.

The finding that $CD4^+CD25^+$ cells have direct effects on $CD25^-$ cells is consistent with the results of Jonuleit et al. and Dieckmann et al. (12, 13). These workers reported that preactivated human $CD4^+CD25^+$ cells induce $CD4^+CD25^-$ cells to become cytokine-producing suppressor cells by a contact-dependent, cytokine-independent mechanism. In this study, the naive $CD4^+CD25^+$ cells were not preactivated, but the costimulatory effects of TGF-β enabled these cells to act on $CD25^-$ cells. The cytokine profile of these cells will be discussed below.

$CD4^+CD25^+$ T_{reg} cells generated ex vivo induce $CD4^+CD25^-$ cells to become T_{reg} cells

Naive $CD4^+$ cells induced to become $CD25^+$ T_{reg} cells with TGF-β also have the capacity to induce other $CD4^+CD25^-$ cells to become T_{reg} cells. $CD4^+CD25^+$ T_{reg} cells were prepared, as described previously, by stimulating naive $CD4^+$ cells with alloantigen and TGF-β1 for 5–6 days, then sorting the $CD25^+$ cells. These TGF-β-conditioned cells, called $T_{reg}1$, have cytokine-independent suppressor activity. Control $CD4^+CD25^+$ cells ($T_{con}1$) were prepared similarly, except that TGF-β was left out of the

cultures. $T_{reg}1$ or $T_{con}1$ $CD25^+$ cells were added to fresh autologous CFSE-labeled $CD4^+CD25^-$ cells in a 1:5 ratio and stimulated with the same alloantigens for another 4–6 days. As expected, $T_{reg}1$ significantly decreased the numbers of $CD4^+$ cells proliferating in response to alloantigens (Fig. 3*A*). The remaining cells showed decreased activation and more cell death in comparison with controls (Fig. 3*B*). Nonetheless, in cultures with $T_{reg}1$ cells, there was an identifiable subset of CFSE-labeled $CD4^+CD25^-$ cells that had divided (Fig. 3*C*).

From these secondary cultures, the different $CD4^+$ cell subsets were obtained by cell sorting and tested for suppressive activity on autologous $CD8^+$ cells. When these alloprimed cells were mixed with fresh autologous T cells in a 1:10 ratio, both $T_{reg}1$ cells and CFSE-labeled $CD4^+CD25^-$ cells that had undergone cell division ($T_{reg}2$) had an equivalent ability to suppress alloactivation of $CD8^+$ cells and the development of CTL activity (Fig. 4*A*). As expected, neither $T_{con}1$ nor CFSE-labeled $T_{con}2$ cells had acquired any suppressive activity.

The ability of $T_{reg}1$ cells to induce $CD4^+CD25^-$ cells to develop suppressive activity was contact dependent. Separation of these two $CD4^+$ T cell subsets from each other by a semipermeable membrane completely abolished their inducer effect (Fig. 4*A*).

FoxP3 *expression by CD4$^+$ regulatory cells induced by IL-2 and TGF-β and by secondarily educated suppressor cells*

The forkhead/winged helix transcription factor *FoxP3* is specifically expressed by T_{reg} cells and programs their development and function (17–19). Using *FoxP3* levels in fresh $CD4^+CD25^+$ and $CD4^+CD25^-$ cells as reference controls, we assessed levels of this transcription factor in $T_{reg}1$ after allostimulation of naive $CD4^+$ cells with IL-2 and TGF-β, and in $T_{reg}2$ educated by $T_{reg}1$ from $CD4^+CD25^-$ precursors. Fig. 5 shows dramatic up-regulation of *FoxP3* mRNA in both of these $CD4^+$ regulatory subsets. *FoxP3* levels in $T_{reg}2$ were even greater than reference control $CD4^+CD25^+$ cells. The TGF-β-induced up-regulation of *FoxP3* in human $CD4^+$ cells is in agreement with the recent findings of Chen et al. (20), who documented that this cytokine increased *FoxP3* gene expression in activated mouse $CD4^+CD25^-$ cells.

The importance of TGF-β and IL-10 in the generation and function of new suppressor cells

Both TGF-β and IL-10 contributed to the generation of $CD4^+$ $T_{reg}2$ cells. In secondary cultures, either anti-TGF-β or anti-IL-10 blocked the ability of $T_{reg}1$ cells to induce $T_{reg}2$ (Fig. 4*B*). In sharp contrast, the ability of $T_{reg}1$ to suppress the proliferative response of $CD8^+$ cells to alloantigens remained intact even in the presence of these neutralizing anti-cytokine Abs.

Unlike $T_{reg}1$ cells, the suppressive activity of $T_{reg}2$ cells was cytokine dependent. We assessed the suppressive activity of these cells on $CD8^+$ cell activation, proliferation, and development of allo-CTL activity. In these studies, the anti-cytokine Abs were added to the tertiary cultures. Fig. 6*A* is representative of 10 experiments in which $T_{reg}2$, in a ratio of 1:10 to responder cells, inhibited $CD8^+$ cell alloactivation by >75%. By contrast, $T_{con}2$ enhanced the number of activated $CD8^+$ cells by >50%. Remarkably, anti-TGF-β and anti-IL-10, used singly and in combination, not only abolished this inhibitory effect, but also enhanced the number of activated $CD8^+$ cells to levels higher than $T_{con}1$. We had previously observed this phenomenon with superantigen-induced $CD4^+$ T_{reg} cells. In that study of T cell-dependent Ab production, neutralization of TGF-β not only abolished suppression, but also significantly enhanced IgG production (9). Taken together, our studies suggest that the T_{reg} we induce with TGF-β are markedly activated and can become helper cells rather than suppressor cells if they lose the capacity to produce these cytokines, or they are neutralized. In support of this hypothesis, lymphocyte production of TGF-β is impaired in systemic lupus erythematosus, and $CD8^+$ T cells from these patients provide B cell help instead of inhibiting Ab production (21, 22).

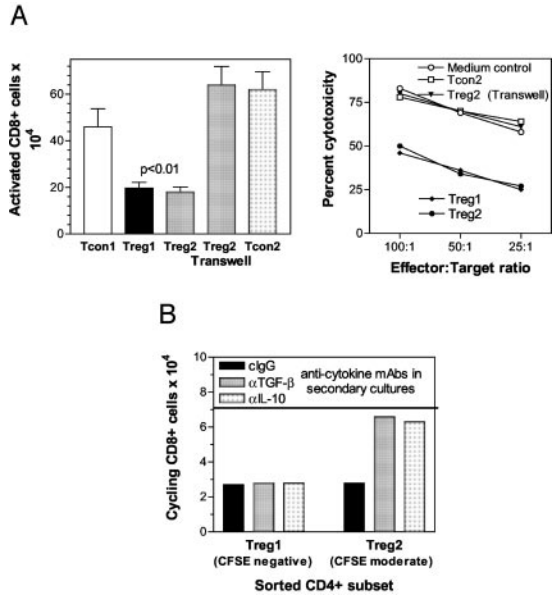

FIGURE 4. $T_{reg}1$ have the ability to induce new $CD4^+CD25^+$ cells to become $T_{reg}2$ through a mechanism that both is contact dependent and requires TGF-β and IL-10. *A*, The generation of $T_{reg}2$ cells is contact dependent. In secondary cultures, $T_{reg}1$ or $T_{con}1$ (>90% $CD25^+$) and CFSE-labeled $CD4^+CD25^-$ cells from donor A were mixed in a 1:10 ratio and cultured with irradiated allostimulator cells from donor B for 5 days. In three of eight experiments, $T_{reg}1$ cells were cultured with stimulator cells in Transwell chambers, where they were separated from responder cells by a semipermeable membrane. At the end of the culture, CFSE-negative $T_{reg}1$, CFSE moderate $T_{reg}2$, CFSE-negative $T_{con}1$, and CFSE moderate $T_{con}2$ were isolated by cell sorting and mixed with CFSE-labeled autologous T cells from donor A in a 1:10 ratio. These cells were restimulated with irradiated non-T cells from donor B for 5–6 days in tertiary cultures. Suppressive activity was assessed by determining the absolute numbers of activated $CD8^+$ ($CD25^+$) cells, or by measuring cytotoxic lymphocyte activity against Con A blasts from donor B in a standard 4-h chromium release assay. *Left panel*, Shows the mean ± SEM of eight experiments; *right panel*, shows the CTL activity of a representative experiment. *B*, The generation of $T_{reg}2$ cells is also cytokine dependent. The effect of anti-TGF-β and anti-IL-10 Abs on the generation of $T_{reg}2$ in secondary cultures was assessed. Anti-cytokine Abs were added in secondary cultures. The suppressive effect of $T_{reg}2$ cells was assessed in tertiary cultures. The experiment shown indicates inhibitory effects on allostimulated, proliferating $CD8^+$ cells. The numbers of CFSE-labeled $CD8^+$ cells that had divided are indicated. Each of the anti-cytokine Abs abolished the generation of new suppressor cells, although the suppressive effects of $T_{reg}1$ were cytokine independent. The result shown is representative of four independent experiments.

The suppressive effects of $T_{reg}2$ cells on the proliferative response of $CD8^+$ cells to alloantigens are also cytokine dependent. The inhibition was reversed by neutralization of TGF-β and IL-10. In this experiment and in most of the six experiments conducted, the inhibitory effects of TGF-β were greater than IL-10. In two experiments, anti-TGF-β, but not anti-IL-10, reversed the inhibition. In some experiments, anti-TGF-β only partially inhibited suppression (Fig. 6*B*). Finally, Fig. 6*C* shows that the development of allo-CTL activity by $CD8^+$ cells is also blocked by $T_{reg}2$, in a cytokine-dependent manner. In this experiment, reversal of the

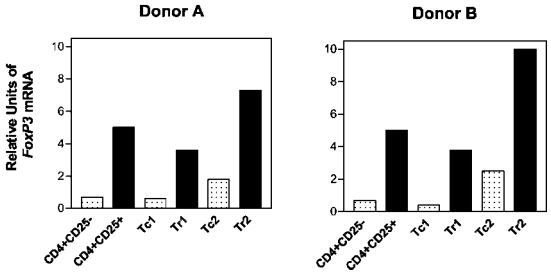

FIGURE 5. Both the natural-like $CD4^+$ regulatory cells induced by IL-2 and TGF-β and the secondarily educated $CD4^+$ cytokine-secreting suppressor cells express FoxP3. Naive $CD4^+$ cells were prepared from donors A and B, and each was stimulated with allogeneic irradiated non-T cells from the respective donors. $T_{reg}1$ or $T_{reg}2$ and $T_{con}1$ or $T_{con}2$ cells from each donor were prepared, as described above. *FoxP3* expression was determined in cDNA samples by a real-time RT-PCR method using GAPDH as an endogenous reference gene.

suppression by anti-cytokine Abs was partial, but in others it was complete.

We considered the possibility that $T_{reg}1$ cells could serve as a source for the TGF-β and IL-10 needed for the generation of $T_{reg}2$. $T_{reg}1$ cells, accordingly, were prepared and restimulated with either T-depleted PBMC cells or beads coated with anti-CD3 and anti-CD28 without TGF-β. Time course studies revealed down-regulation of IL-2 production by day 3, poor IFN-γ production at all times, and an increase in IL-10-producing cells beginning at day 3 (Fig. 7*A*). The flow cytometry profile at day 3 is shown in Fig. 7*B*. Not shown is an absence of IL-4 and no difference between TNF-α production between $T_{reg}1$ and $T_{con}1$ cells.

Separation of $T_{reg}1$ and $T_{con}1$ cells into $CD25^+$ and $CD25^-$ subsets by cell sorting revealed that by day 5 $T_{reg}1$ $CD25^+$ cells produced IL-10 and high levels of TGF-β in the biologically active form (Fig. 7*C*). Remarkably, this was the only T cell subset that produced these cytokines. These studies, therefore, provide evidence that $T_{reg}1$ can produce the cytokines needed for the induction of new $CD4^+$ suppressor cells.

This study may help to resolve apparently conflicting observations regarding the role of cytokines in the suppressive activities of $CD4^+CD25^+$ cells isolated from lymphoid tissues. Although some workers have reported that suppression is independent of TGF-β and IL-10, others have claimed that suppression can be reversed by anti-cytokine-neutralizing Abs, especially at high concentrations (23). It has become evident that $CD4^+CD25^+$ T_{reg} cells constitute heterogeneous populations. Our studies support the view that natural $CD4^+$ T_{reg} cells have a cytokine-independent mechanism of action (1–3), but that $CD4^+CD25^+$ T_{reg} cells induced in the periphery may have either cytokine-dependent effects or cytokine-independent effects.

An important new finding is that the target of the costimulatory effects of TGF-β are $CD4^+CD25^+$ cells. Whereas previously we had reported that IL-2 and TGF-β enhance expression of CD25, CTLA-4, and CD40L (8, 24), in this study we demonstrate that expression of CD122 and GITR is also enhanced. Depletion of the few $CD25^+$ cells found in the naive fraction of $CD4^+$ cells completely abolished these costimulatory effects.

Remarkably, the effects of IL-2 and TGF-β on $CD4^+CD25^+$ cells did not significantly expand these cells in vitro, but stimulated the expansion of $CD4^+CD25^-$ cells and induced them to become suppressor cells. We and others have reported that $CD4^+CD25^-$ cells activated in the presence of TGF-β become T_{reg} cells (9, 25). In this study, naive, natural $CD4^+CD25^+$ cells treated with IL-2 and TGF-β greatly amplified the effect of these cytokines on

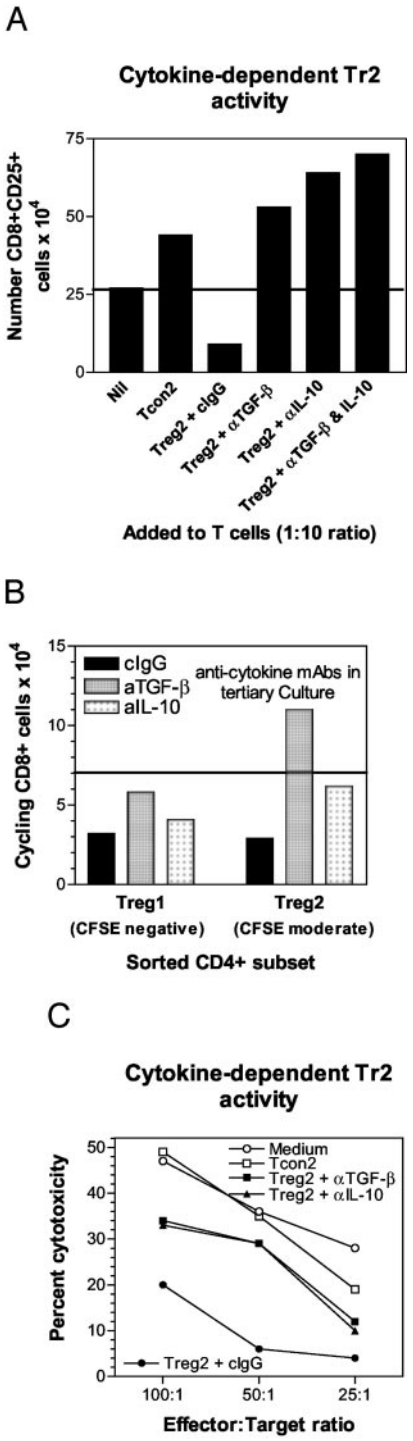

FIGURE 6. $T_{reg}2$ cells have a cytokine-dependent mechanism of action. *A*, Suppressive effects on alloactivated $CD8^+$ cells. The experimental design was described in the legend for Fig. 2. Sorted $T_{reg}2$ cells from secondary cultures were mixed with CFSE-labeled autologous T cells in a 1:10 ratio and restimulated with alloantigen for 5 days. From cell counts and the percentage of $CD8^+CD25^+$ cells by FACS, the absolute numbers of activated $CD8^+$ are shown. The baseline (labeled as Nil) is determined by $CD8^+CD25^+$ cells from the MLR without added primed T cells. The effect of anti-TGF-β and anti-IL-10 added singly and together is shown. The result shown is representative of 10 independent experiments. *B*, Suppressive effects on proliferating $CD8^+$ cells. The experimental design is the same as that described in Fig. 3*A*. The difference is that the anti-cytokine Abs were added to tertiary cultures. *C*, Suppressive effects on the generation of CTL activity. The effects of $T_{reg}2$ or $T_{con}2$ cells on the ability of T cells to develop allo-CTL activity are shown in a standard 4-h chromium release assay at various E:T cell ratios.

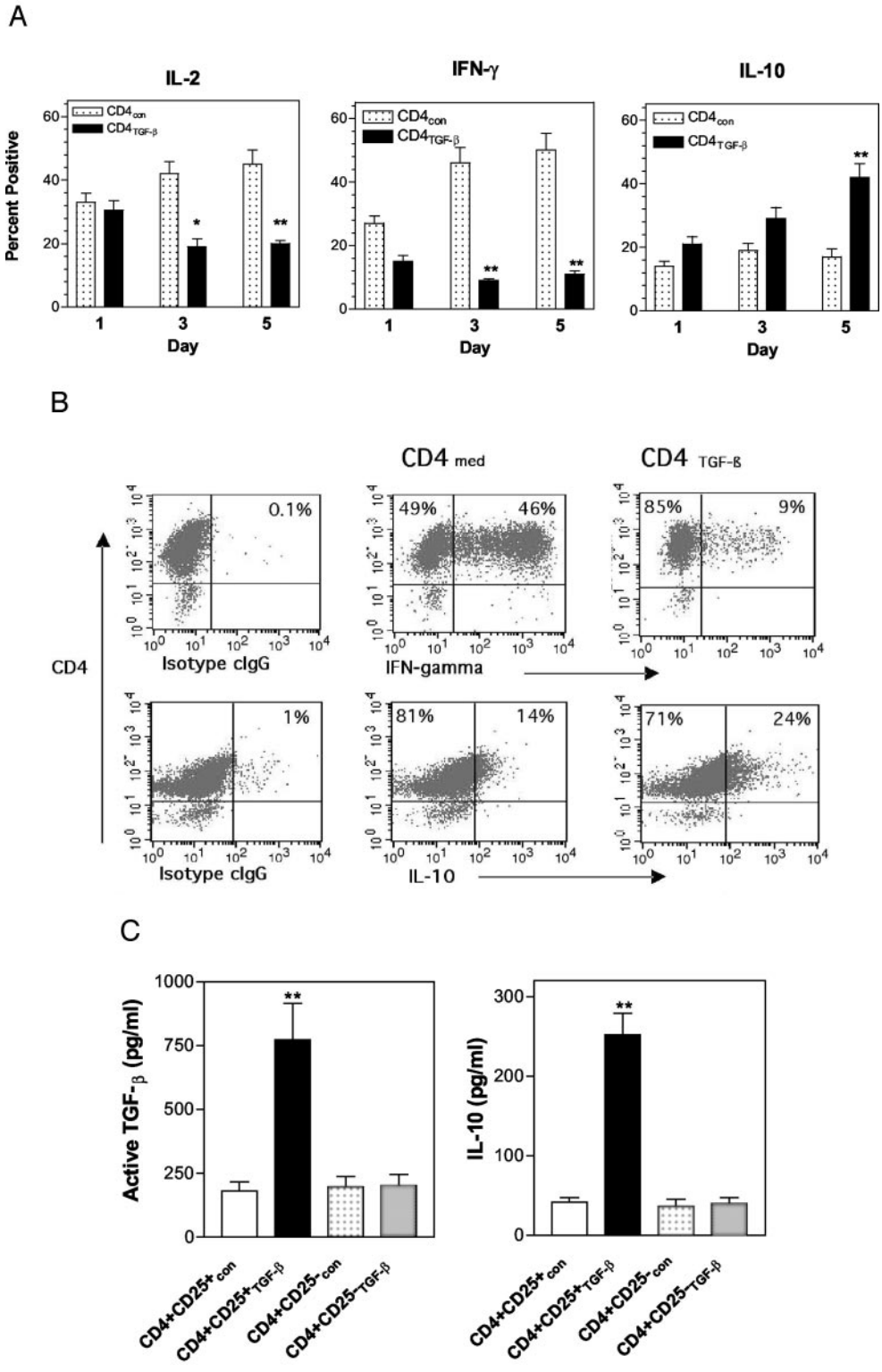

FIGURE 7. Cytokine profile of $T_{reg}1$ cells. *A*, Naive $CD4^+$ cells from donor A were stimulated with irradiated non-T cells for donor B with IL-2 (10 U/ml) ± TGF-β (10 ng/ml) for 6 days. The primed $CD4^+$ cells were restimulated with allogeneic cells, IL-2, but without TGF-β. At various times (days 1, 3, and 5), the capacity of these cells to produce IL-2, IFN-γ, and IL-10 was determined by stimulating the cells with PMA and ionomycin for 6 h and intracellular staining of permeabilized cells, as described in *Materials and Methods*. In comparison with control alloprimed (*Figure legend continues*) naive $CD4^+$ cells, those primed with TGF-β had a decreased capacity to produce IL-2 by day 3, an inability to produce IFN-γ, but an enhanced ability to produce IL-10. IL-4 was also measured, but was undetectable. *B*, FACS analysis of IFN-γ and IL-10 production at day 3. The example shown is representative of 10 separate experiments. *C*, TGF-β and IL-10 production is restricted to $CD4^+CD25^+$ cells. After primary cultures, $CD4^+$ cells were sorted into $CD25^+$ $T_{reg}1$ cells and $T_{con}1$ cells and their respective $CD25^-$ subsets. Each was restimulated with anti-CD3- and anti-CD28-coated beads for 5 days, and the cell supernatants were tested for TGF-β and IL-10 production.

$CD4^+CD25^-$ cells, thus increasing the numbers of T_{reg} generated. Although their suppressive effects were also cytokine independent in vitro, upon restimulation they produced both TGF-β and IL-10, and these cytokines had an important role in the generation of new T_{reg} cells.

The next new finding is that $CD4^+CD25^+$ $T_{reg}1$ cells could induce other $CD4^+CD25^-$ cells to become suppressor cells. Unlike the generation of $T_{reg}1$ cells that required exogenous cytokines for their development, the TGF-β and IL-10 produced by $T_{reg}1$ were sufficient for the alloactivated $CD4^+CD25^-$ cells to become $T_{reg}2$ cells. Although the generation of $T_{reg}2$ cells required cell contact, the presence of TGF-β and IL-10 was also necessary for this phenomenon. Physical interaction between the $CD4^+CD25^+$ inducer and $CD4^+CD25^-$ cells was also required for their differentiation to suppressor cells (12, 13). In our recent study, in which we induced $CD4^+CD25^-$ cells to become TGF-β-producing suppressor cells, their suppressive activity was also contact dependent, but nonetheless abolished

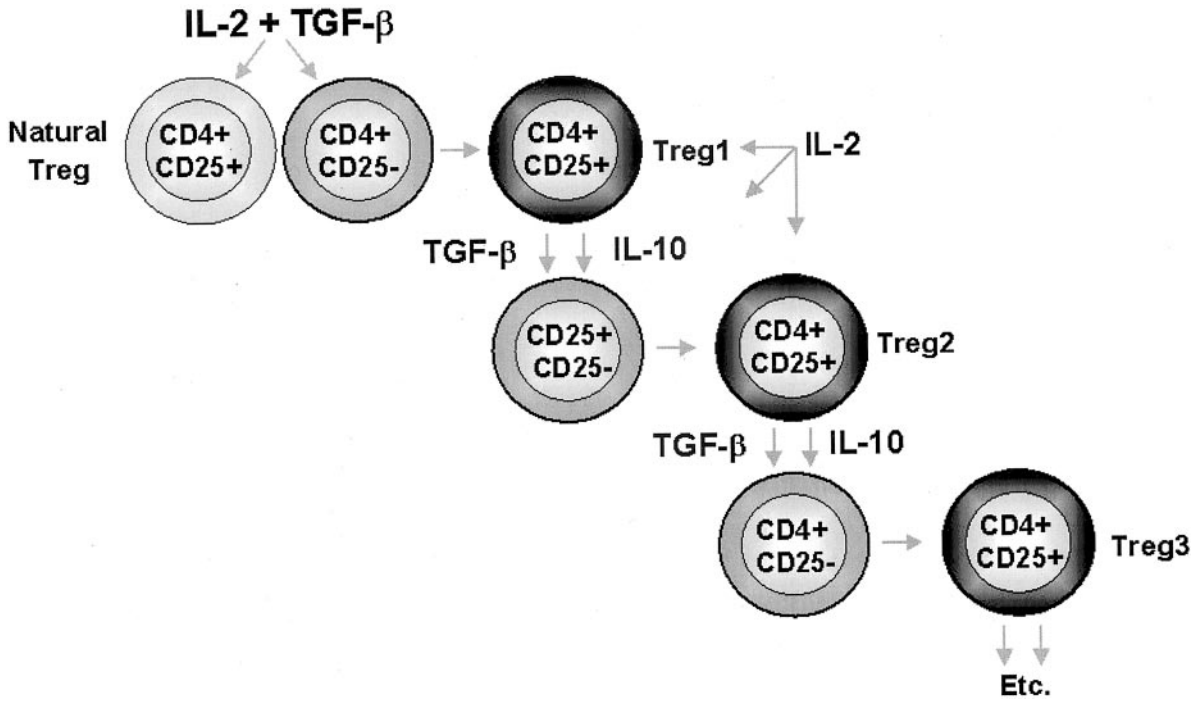

FIGURE 8. A model for the effects of IL-2, TGF-β, and IL-10 in a circuit for the sustained renewal of $CD4^+$ T_{reg} cells. IL-2 and TGF-β induce Ag-activated $CD4^+CD25^-$ cells to become $CD4^+CD25^+$ $T_{reg}1$. These cytokines also costimulate natural $CD4^+CD25^+$ cells that do not proliferate, but greatly expand the developing $CD4^+CD25^+$ $T_{reg}1$ that have a contact-dependent, cytokine-independent mechanism of action. Restimulated $T_{reg}1$ also produce both TGF-β and IL-10 and induce other Ag-activated $CD4^+CD25^-$ cells to become $T_{reg}2$ by a contact-dependent mechanism that also requires both of these cytokines. Ag-activated $T_{reg}2$ also has the ability to produce TGF-β and IL-10 and can induce other $CD4^+CD25^-$ cells to become $T_{reg}3$, which can also induce $T_{reg}4$, etc. This circuit depends upon repeated exposure of Ag, and IL-2 produced by Ag-stimulated T cells. It probably also depends upon specialized APCs that remain to be characterized.

by anti-TGF-β (9). Others have also reported that surface-bound TGF-β may mediate the suppressive effects of $CD4^+CD25^+$ cells (23).

In this study, we added TGF-β to $CD4^+$ cells to initiate T_{reg} differentiation, but in vivo both TGF-β and IL-10 are produced by cells of the innate immune system. It is likely that in the gastrointestinal tract, the respiratory tract, and the female reproductive system, immature APCs produce these cytokines that drive Ag-activated T cells in these organs to become cytokine-producing suppressor cells analogous to those we generated ex vivo. A model suggesting that the effects of IL-2 and TGF-β on both natural $CD4^+CD25^+$ cells and $CD4^+CD25^-$ cells trigger a continuous loop that sustains the renewal of $CD4^+CD25^+$ T_{reg} cells is shown in Fig. 8. IL-2 and TGF-β costimulate Ag-activated natural T_{reg} cells, and these cells, together with these two cytokines, induce naive $CD4^+CD25^-$ cells to become suppressor cells that can produce IL-10 and the active form of TGF-β. The stimulated $CD4^+CD25^-$ cells, therefore, produce the cytokines that have previously been shown to be involved in the generation of T_{reg} cells (4–9). The TGF-β- and IL-10-producing $CD4^+CD25^+$ T_{reg} cells are then able to induce other Ag-activated $CD4^+$ cells to become T_{reg} cells. The sustained renewal of T_{reg} cells that express FoxP3 would insure the maintenance of nonresponsiveness to self Ags and exogenous Ags presented by mucosal APCs.

We have recently reported that a single transfer of T_{reg} cells generated ex vivo with TGF-β and IL-2 will double the survival of mice that had developed a lupus-like syndrome (26). We suggest that the long-term effects of adoptively transferred natural-like $CD4^+CD25^+$ regulatory cells induced ex vivo are due to their ability to generate new cytokine-producing $CD4^+$ T_{reg} cells in vivo. Our studies raise the possibility, therefore, that the adoptive transfer of $CD4^+$ T_{reg} cells generated ex vivo with IL-2 and TGF-β as a treatment for autoimmune diseases may have sustained long-term beneficial effects in vivo.

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