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A Role for TGF-β in the Generation and Expansion of $CD4^+CD25^+$ Regulatory T Cells from Human Peripheral Blood[1]

Satoshi Yamagiwa,[2] J. Dixon Gray, Shigeo Hashimoto, and David A. Horwitz[3]

An elusive goal in transplanting organs across histocompatibility barriers has been the induction of specific tolerance to avoid graft rejection. A considerable body of evidence exists that the thymus produces regulatory T cells that suppress the response of other T cells to antigenic stimulation. We report that TGF-β can induce certain $CD4^+$ T cells in the naive ($CD45RA^+RO^-$) fraction in human peripheral blood to develop powerful, contact-dependent suppressive activity that is not antagonized by anti-TGF-β or anti-IL-10 mAbs. The costimulatory effects of TGF-β on naive $CD4^+$ T cells up-regulated CD25 and CTLA-4 expression, increased their transition to the activated phenotype, but decreased activation-induced apoptosis. Suppressive activity was concentrated in the $CD25^+$ fraction. These $CD4^+CD25^+$ regulatory cells prevented $CD8^+$ T cells from proliferating in response to alloantigens and from becoming cytotoxic effector cells. Moreover, these regulatory cells exerted their suppressive activities in remarkably low numbers and maintained these effects even after they are expanded. Once activated, their suppressive properties were Ag nonspecific. Although <1% of naive $CD4^+$ T cells expressed CD25, depletion of this subset before priming with TGF-β markedly decreased the generation of suppressive activity. This finding suggests that $CD4^+CD25^+$ regulatory T cells induced ex vivo are the progeny of thymus-derived regulatory T cells bearing a similar phenotype. The adoptive transfer of these regulatory T cells generated and expanded ex vivo has the potential to prevent rejection of allogeneic organ grafts. ***The Journal of Immunology*****, 2001, 166: 7282–7289.**

Considerable evidence has accumulated for the existence of regulatory T cells that prevent autoimmunity and maintain transplantation tolerance (1, 2). One regulatory subset that has been well characterized expresses cell surface IL-2R α-chains (3, 4). These $CD4^+CD25^+$ T cells differentiate in the thymus and are exported to the periphery, where they suppress the activation of potentially self-reactive cells (3–8). Injection of peripheral T cells from normal mice depleted of $CD4^+CD25^+$ T cells into athymic mice results in a high incidence of organ-specific autoimmune disease (3, 4). Moreover, certain strains of neonatally thymectomized mice develop multiorgan-specific autoimmunity (9, 10). These mice lack $CD4^+CD25^+$ cells because they are not produced until 1 wk after birth (11, 12).

$CD4^+CD25^+$ T cells are potent inhibitors of polyclonal T cell activation (12). After activation via the TCR, they inhibit IL-2 production by the responding T cells (13–15). Unlike other regulatory T cells, which produce inhibitory cytokines (16, 17), these cells suppress immune responses by a contact-dependent mechanism, at least in vitro (15). Others have described $CD4^+CD25^+$ cells that regulate anergy in neonatally tolerized mice (18).

Whether regulatory $CD4^+CD25^+$ T cells exist in humans and can be expanded in the periphery is not known. In addition to the well-described suppressive effects of TGF-β on T cell function (19–24), this cytokine can also have positive effects, which are predominantly on naive T cells (25). TGF-β can synergize with IL-2 to prevent apoptosis and promote effector cell function (26–28), and it has a crucial role in the generation of $CD8^+$ T cells that suppress Ab production (29, 30). Here we report that TGF-β also induces certain naive $CD4^+$ T cells to become cells that suppress the generation of T cell cytotoxic activity. Using the allogeneic mixed lymphocyte reaction to stimulate naive $CD4^+$ cells that express 1% IL-2R α-chains, we have learned that TGF-β enhances the number of $CD25^+$ cells and that some of these T cells inhibit $CD8^+$ cells from proliferating and developing cytolytic activity on restimulation. Surprisingly, depletion of the rare $CD25^+$ cells in the $CD4^+CD45RA^+RO^-$ fraction markedly reduced the generation of suppressive activity; a result suggesting that TGF-β stimulates and expands the small number of thymus-derived $CD4^+CD25^+$-regulatory T cells that circulate in peripheral blood.

Materials and Methods

Reagents

Antibodies used were anti-CD3 (UCHT1; PharMingen, San Diego, CA); anti-CD4 (OKT4; American Type Culture Collection (ATCC), Rockville, MD); anti-CD8 (OKT8; ATCC); anti-CD11b (OKM1; ATCC); anti-CD16 (3G8); anti-CD25 (PharMingen); anti-CD45RA (PharMingen); anti-CD45RO (UCHL-1; ATCC); anti-CD69 (PharMingen); anti-CD74 (anti-HLA-DR) (L243, ATCC); anti-CD95 (Fas/APO-1) (PharMingen); anti-CD152 (CTLA-4; PharMingen). FITC-conjugated annexin V and anti-IL-10 were also purchased form PharMingen. Human rIL-2 was purchased from Chiron (Emeryville, CA). Human rTGF-β1, anti-TGF-β, and IL-10 were purchased from R&D Systems (Minneapolis, MN).

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Cell isolation

PBMC were prepared from heparinized venous blood of healthy adult volunteers by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation as previously described (31). The PBL were immediately rosetted with 2-aminoethylisothiouronium bromide-treated SRBC. T cells were prepared from rosetting cells by negative selection following depletion of $CD16^+$ and $CD74^+$ cells using immunomagnetic beads (Dynal, Great Neck, NY). The percentage of $CD3^+$ cells in this fraction was usually >95%. To obtain naive $CD45RA^+RO^-CD4^+$ T cells, SRBC-rosetting cells were stained with mAbs to CD8, CD16, CD11b, CD74, and CD45RO and separated by negative selection using immunomagnetic beads. The same mAbs were used to obtain naive $CD45RA^+RO^-CD8^+$ T cells except that CD4 was substituted for CD8. The purity of those T cells was usually>95%. In some experiments, $CD25^+$ cells were also depleted by adding anti-CD25 to the panel of mAbs. In other experiments, after naive $CD4^+$ cells had been cultured with irradiated allogeneic stimulators ± TGF-β1 (1 ng/ml) for 5 days, the $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets were obtained by sorting on a FACStar^{Plus} flow cytometer (Becton Dickinson, San Jose, CA) using FITC-conjugated anti-CD25 and PE-conjugated anti-CD4. The resultant purity was >98%. The nonrosetted cells from each donor were frozen and thawed for use as stimulator cells in a subsequent experiment.

Cell cultures

Purified naive $CD4^+$ T cells (10^6 cells/ml) were added to 24-well microtiter plates (Falcon, Lincoln Park, NJ) in 2-ml volumes. Cells were suspended in AIM-V serum-free lymphocyte medium (Life Technologies, Gaithersburg, MD) because serum contains significant amounts of latent TGF-β. Cells were stimulated with irradiated (3000 cGy) allogeneic T cell-depleted PBMC (10^6 cells/ml) in the presence or absence of TGF-β (0.1–1 ng/ml) for 5 days. The phenotype of these alloactivated $CD4^+$ cells compared with unstimulated $CD4^+$ cells was assessed by flow cytometry. After washing to remove residual cytokines, the regulatory effects of these $CD4^+$ subsets on syngeneic effector T cells was assessed in secondary cultures. These cultures consisted of freshly isolated effector T cells stimulated for 5 days with irradiated allogeneic T cell-depleted PBMC in the presence or absence of the indicated numbers of primed $CD4^+$ T cells. In some experiments, various numbers of sorted (see above) $CD4^+CD25^+$ or $CD4^+CD25^-$ were added to the secondary cultures. We assessed effector T cell phenotype, proliferative activity, and generation of CTL activity. In some secondary cultures, the medium contained 10% AB human serum (Omega Scientific, Tarzana, CA) instead of FCS.

Similar to the studies of Thornton and Shevach (13), we used the Transwell chambers (Corning Costar, Cambridge, MA) to assess whether surface contact was necessary for suppressive effects in the secondary cultures. These experiments were conducted in 24-well plates (0.8 ml) with effector T cells and irradiated (3000 cGy) allogeneic T cell-depleted PBMC in the presence or absence of conditioned $CD4^+$ T cells mixed with irradiated stimulator cells in the Transwell. In other experiments, sorted $CD4^+CD25^+$ T cells were cultured with IL-2, 10 U/ml, for 5–7 days to determine their capacity for expansion. Various numbers of these cells were added to fresh T cells and irradiated stimulator cells, and their effect on the generation of CTL activity was assessed as described above.

Cytotoxicity assays

The cytotoxic activity was measured by a standard 4 h chromium release assay. In brief, 5×10^3 ^{51}Cr-labeled target cells (stimulator Con A blasts) were incubated with graded numbers of effector T cells in 200 μl medium in 96-well round-bottom microtiter plates (Falcon). The plates were centrifuged and incubated for 4 h at 37°C. Then supernatants were harvested and counted in a gamma counter. All test samples were measured in triplicate. The percentage of specific ^{51}Cr release was calculated as: percent lysis = [(cpm released experimental − cpm spontaneous)/(cpm total lysis − cpm spontaneous)] × 100.

Proliferation assays

Purified T cells (10^5 cells/well) were cultured in 200 μl flat-bottom 96-well plates (Falcon) with irradiated (3000 cGy) allogeneic T cell-depleted PBMC (10^5 cells/well) and the indicated numbers of conditioned $CD4^+$ T cell for 3 or 5 days at 37°C and 5% CO_2. Cultures were pulsed with [^{3}H]TdR for the last 18 h of culture. All tests were conducted in triplicate.

Immunofluorescence analysis

Cell surface Ag expressions on both conditioned $CD4^+$ T cells and effector T cells were determined by immunofluorescence. Cells (10^5) were labeled with FITC-conjugated (anti-CD4, anti-CD25, anti-CD45RO, anti-CD69), PE-conjugated (anti-CD8, anti-CD25, anti-CD45RA, anti-CD95) or CyChrome-conjugated (anti-CD4, anti-CD8) mAbs. Cells were incubated with the appropriate mAbs for 30 min at 4°C in PBS with 0.1% BSA and 0.02 mM NaN_3. After washing, the labeled cell samples were analyzed on a FACStar^{Plus} flow cytometer using Cellquest software (Becton Dickinson).

To analyze cell divisions of effector T cells, effector T cells were labeled with CFSE (Molecular Probes, Eugene, OR). In brief, cells were washed and resuspended at a concentration of 10^7/ml in PBS. CFSE was added at a final concentration of 0.5 μM and incubated for 10 min at 37°C. The reaction was stopped by washing the cells with RPMI 1640 containing 10% FCS. After the culture of labeled effector T cells (10^6 cells/ml) with irradiated (3000 cGy) allogeneic T cell-depleted PBMC (10^6 cells/ml) in the presence or absence of the indicated numbers of conditioned $CD4^+$ T cells, CFSE levels were analyzed by flow cytometry.

To learn whether $CD4^+$ T cells primed with TGF-β could proliferate in response to the stimulator cells, these T cells and those primed without TGF-β were rested for 3 days, labeled with CFSE, and restimulated with irradiated E rosette-negative cells in medium containing 10% type AB human serum. Intensity of CFSE was determined after 3–7 days of culture.

Statistical analysis

The significance of the results was analyzed by Student's *t* test performed using GraphPad software (GraphPad, San Diego, CA).

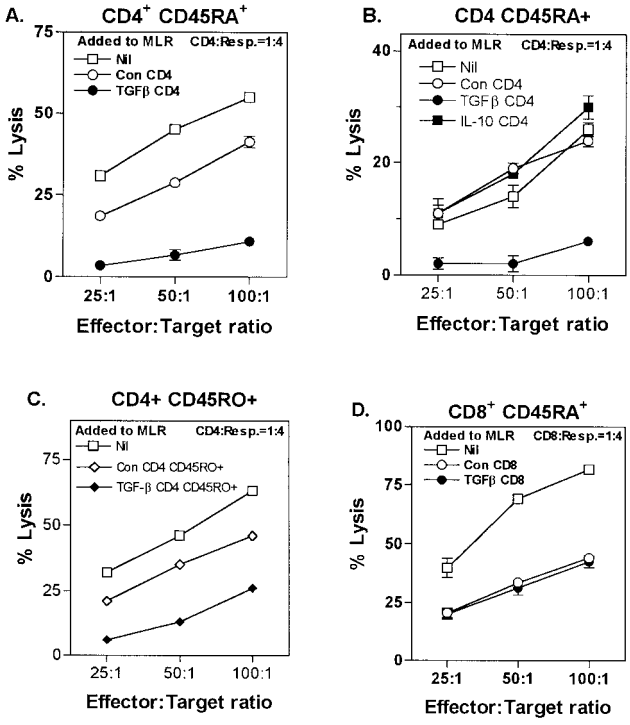

FIGURE 1. Generation of potent suppressive activity from naive $CD4^+$. Generation of in a two-step coculture procedure, purified T cell subsets from donor A were stimulated for 5 days with irradiated (3000 cGy) allogeneic stimulator cells from donor B ± TGF-β (1 ng/ml). The secondary cultures consisted of mixing the primed T cell subsets with fresh syngeneic T cells in the ratio indicated, and culturing these cells with irradiated stimulator cells from donor B for 5 days. CTL activity against Con A blasts from donor B was then determined in a standard 4-h chromium release assay at the E:T ratios indicated. *A,* Alloactivated $CD4^+$ $CD45RA^+RO^-$ cells primed with TGF-β markedly suppressed the generation of CTL activity in comparison with control (Con) $CD4^+$ cells. Additional controls not shown were $CD4^+$ cells cultured for 5 days with TGF-β, but without allogeneic MHC stimulation. These cells had no suppressive effects. *B,* Under similar experimental conditions, rIL-10 (10 pg/ml) was unable to induce suppressive activity. *C,* Suppressive effects of $CD4^+CD45RA^-RO^+$ cells primed with allogeneic stimulator cells. *D,* Suppressive effects of $CD8^+CD45RA^+RO^-$ cells. TGF-β did not enhance the inhibitory effects of primed $CD8^+$ cells. Resp., responder cells.

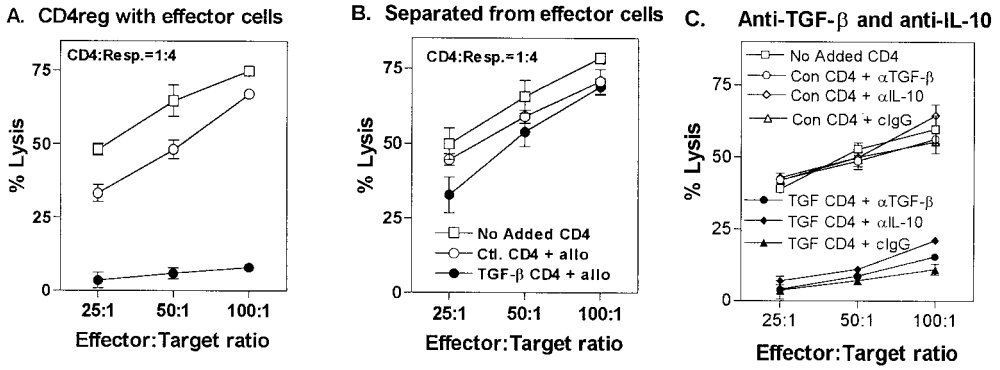

FIGURE 2. Suppressive effects of CD4reg require cell to cell contact. *A*, CD4reg and control CD4$^+$ cells were generated by activating naive CD4$^+$ cells with allogeneic stimulator cells (allo) ± TGF-β as described above. *A*, In secondary cultures, CD4reg or control CD4$^+$ cells were directly mixed with the responder cells (Resp.); *B*, CD4$^+$cells were separated from the responder T cells by a semipermeable membrane using Transwell chambers. The Transwells containing the CD4$^+$ cells also included stimulator cells. Results are one of three independent experiments. *C*, Effects of anti (α)-TGF-β, anti-IL-10 and isotype-matched control IgG (all Abs were added at 10 mg/ml). One of two experiments is shown.

Results

TGF-β can induce alloactivated naive CD4$^+$ T cells to develop suppressive activity

Naive CD4$^+$ cells developed potent suppressive activity when activated in the presence of TGF-β. These CD4$^+$ T cells had the capacity to block the generation of CTL activity against allogeneic target cells. In a two-step coculture experiment, CD4$^+$CD45RA$^+$RO$^-$ T cells were first incubated with irradiated allogeneic stimulator cells ± TGF-β1 for 5 days. Various numbers of these cells were then added to fresh T cells and stimulator cells, and the effect of these cells on the generation of CTL activity was assessed. On assay with chromium-labeled allogeneic target cells, CD4$^+$ cells that had been primed in the presence of TGF-β almost completely blocked responder T cells from killing, whereas control CD4$^+$ cells generally had minimal to modest suppressive activity (Fig. 1*A*). Activation of CD4$^+$ cells was needed for the development of inhibitory activity. CD4$^+$ suppressor cells that had been primed with alloantigens in the presence of TGF-β will be called CD4reg. Unlike TGF-β, priming of the CD4$^+$ cells with IL-10 did not induce suppressive activity (Fig. 1*B*). TGF-β could also costimulate purified CD4$^+$CD45RA$^-$CD45RO$^+$ cells to inhibit allo-CTL activity (Fig. 1*C*). However, the effects of these cells on the development of T cell cytotoxicity were more variable. In some experiments, the effects of CD4 cells primed without TGF-β (CD4con) were also strongly suppressive, and the presence of TGF-β did not further increase this effect. Finally, naive CD8$^+$ T cells primed with stimulator cells also developed moderate suppressive activity, but under these experimental conditions the presence of TGF-β with few exceptions, did not enhance this activity (Fig. 1*D*),

Similar to the murine CD4$^+$-regulatory T cells described by Shevach et al. (6, 11–13), suppression by TGF-β-induced human CD4$^+$ cells alloactivated in the presence of TGF-β appeared to require surface contact (Fig. 2, *A* and *B*). Separation of these cells from responder T cells by a semipermeable membrane completely prevented their inhibitory effects. This was the case even if they were activated with stimulator cells. To learn whether surface contact induced the production of inhibitory cytokines, experiments were performed with mAbs that neutralized TGF-β and IL-10.

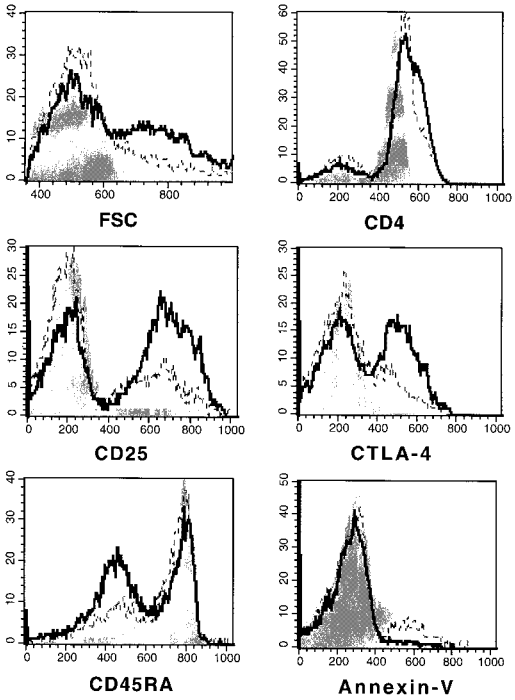

FIGURE 3. Activation of naive CD4$^+$ T cells in the presence of TGF-β enabled them to respond more vigorously to alloantigens, accelerated their conversion to the activated phenotype, and increased their viability. Purified naive CD4$^+$ T cells were cultured for 5 days without stimulator cells (gray shaded area), with irradiated allogeneic stimulator cells (thin dotted line), or with stimulator cells and TGF-β (1 ng/ml; thick line) and expression of cell surface or intracellular Ags were determined by flow cytometry. Cells (10^5) were labeled with FITC-conjugated (anti-CD4, anti-CD25), PE-conjugated (anti-CD45RA, anti-CTLA-4), or CyChrome-conjugated (anti-CD4) mAbs. CTLA-4 expression was analyzed by intracellular staining. To detect apoptotic cells, annexin V staining was performed according to the manufacturer's instructions. This experiment is representative of at least five studies with each marker. FSC, Forward scatter.

Table I. *Costimulatory effects of TGF-β on alloactivated naive T cells*[a]

	Control (%)	TGF-β (%)	*p*
CD25	34.5 ± 3.8	42.3 ± 3.6	<0.01
CTLA-4			
Intracellular	23.2 ± 4.5	31.5 ± 4.1	<0.01
Surface	5.2 ± 0.4	10.8 ± 0.9	<0.01

[a] CD45RO-depleted CD4$^+$ cells were stimulated with irradiated allogeneic non-T cells for 5 days with or without TGF-β and stained for the markers indicated as described in *Materials and Methods*. *p* values were determined using the paired *t* test. $N = 13$.

These Abs, however, did not diminish the suppressive effects of CD4reg (Fig. 2*C*). Irradiation of CD4reg with 3000 cGy after they had been primed with TGF-β, however, completely abolished suppressive activity (result not shown).

Costimulatory effects of TGF-β on alloactivated CD4$^+$ T cells

Activation of naive CD4$^+$ T cells in the presence of TGF-β enabled them to respond more vigorously to alloantigens, accelerated their conversion to the activated phenotype, and increased their viability. Fig. 3 indicates properties of control and TGF-β-conditioned CD4$^+$ cells after 5 days of culture. Allostimulated CD4$^+$ cells displayed the typical features of activation and some were already undergoing activation-induced cell death as indicated by annexin V staining. Importantly, those alloactivated in the presence of TGF-β were even larger, some stained more intensely with CD4, and expression of CD25 was markedly increased. Table I shows the significant increase in mean values for expression of CD25 and for both intracellular and surface expression of CTLA-4 in 13 separate experiments. A greater percentage of CD4reg had also down-regulated surface expression of CD45RA (Fig. 3). Significantly, annexin V staining was markedly decreased, and the total number of CD4reg recovered was 50–60% greater than control CD4$^+$ cells. Thus, although CD4reg were more intensively activated that control CD4 cells, they were also more resistant to activation-induced apoptosis.

CD4$^+$ regulatory T cells are CD25$^+$

Similar to the murine CD4$^+$-regulatory T cells described by others (11–15), separation of CD4reg into CD25$^+$ and CD25$^-$ fractions by cell sorting revealed that almost all of the suppressive activity was contained in the CD25$^+$ fraction (Fig. 4*A*). The CD4$^+$CD25$^-$ fraction displayed only minimal suppressive activity. Moreover, the CD4$^+$CD25$^+$ cells were very potent as indicated by their ability to markedly inhibit the generation of CTL activity in very low numbers. Fig. 4*B* shows that suppressive activity was only slightly diminished when the numbers of the CD4$^+$ suppressor cells were reduced from 25% to 3% of total T cells. With this small number of added CD4$^+$ cells, the minimal suppressive activity mediated by the CD25$^-$ subset had disappeared.

CD4$^+$CD25$^+$ regulatory T cells can proliferate in response to alloantigens

Unlike CD4$^+$-regulatory T cells generated by repeated stimulation with IL-10, which have low proliferative capacity (32), CD4reg induced in the presence of TGF-β proliferated in response to alloantigens and IL-2 and retained their suppressive capacity. Fig. 5 shows the response of CD4reg and CD4con when cultured with stimulator cells. After priming, the cells were washed to remove residual TGF-β and cultured for 3 days; CFSE labeling revealed that they had returned to the resting state. After the addition of allogeneic stimulator cells to CD4$^+$ cells freshly labeled with CFSE, a stronger proliferative response by CD4reg was apparent at day 3 and clearly evident by day 5.

In other experiments, the CD25$^+$ fraction of CD4reg was isolated by cell sorting and cultured for 5 days with IL-2. These cells expanded 7- to 14-fold during this time and retained their suppressive function in three separate experiments. Fig. 6 shows strong inhibition of the generation of CTL activity when these CD4reg comprised only 5% of the total T cells. Moreover, this suppressive activity persisted when the number of these CD4$^+$CD25$^+$ cells was reduced to only 0.2% of total cells. Irradiation of these regulatory T cells, however, abolished their suppressive effects.

Mechanism of action of CD4$^+$CD25$^+$ regulatory T cells

CD4reg blocked the proliferative response of responder T cells to alloantigens (Fig. 7*A*). Gating on CD8$^+$ cells by flow cytometry after a 5-day culture, these alloactivated T cells displayed the expected marked increase in CD25, increased expression of CD69 and Fas (CD95), and evidence of cell division by CFSE-labeled and propidium iodide-labeled effector T cells. Moreover, some CD8$^+$ cells were undergoing activation-induced cell death as indicated by annexin V staining. In sharp contrast, in cultures containing CD4reg, up-regulation of CD25, CD69, and Fas by CD8$^+$ cells was markedly inhibited, and almost none underwent apoptosis or cell division. Studies of the absolute numbers of CD8$^+$ cells in culture revealed only a minimal change from the total count. Thus, CD4reg prevented CD8$^+$ T cells from responding to alloantigens.

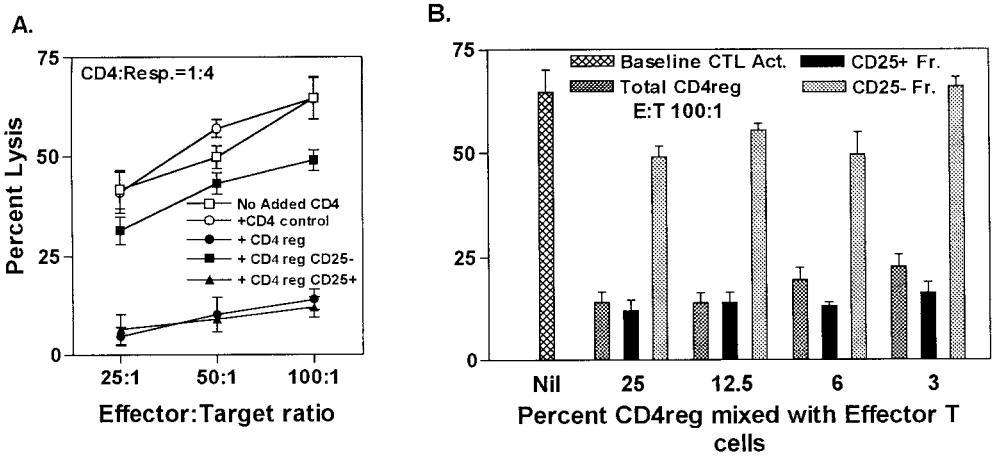

FIGURE 4. CD4reg display CD25 and require remarkably small numbers for potent suppressive activity. Naive CD4$^+$ T cells primed with irradiated allogeneic stimulator cells ± TGF-β (1 ng/ml) and CD4reg were separated into CD25$^+$ and CD25$^-$ fractions (Fr.) by cell sorting. *A*, Effect of primed CD4$^+$ cells mixed with fresh T cells at a 1:4 ratio. The experimental protocol described in Fig. 1 was used to assess regulation of the generation of CTL activity. *B*, Effect of various dilutions of these primed CD4$^+$ T cells added to fresh responder cells (Resp.) on the generation of CTL activity at an E:T ratio of 100:1. Results shown are representative of three similar experiments.

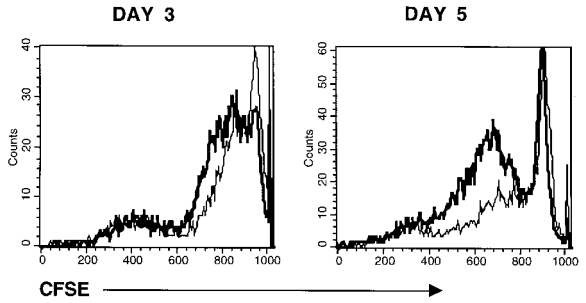

FIGURE 5. CD4reg can proliferate in response to alloantigens. Naive $CD4^+$ T cells and equal numbers of irradiated stimulator cells (1×10^6/ml) were cultured ± TGF-β for 5 days in serum-free medium. After a washing to remove residual TGF-β, the cells were rested for 3 days in culture medium containing 10% normal human serum. The cells were then labeled with CFSE and restimulated with irradiated allogeneic stimulator cells. The intensity of CFSE staining after 3 and 5 days of culture is shown. Heavy line, CD4reg; thin line, CD4con. Similar results have been obtained in four other experiments.

Other studies were conducted to learn the Ag specificity of CD4reg. After priming naive $CD4^+$ T cells from donor A with stimulator cells from donor B, these activated T cells were mixed with autologous T cells and stimulator cells from donor B or from a third party, donor C. The effects of these $CD4^+$ cells on the generation of CTL activity against chromium-labeled cells from the same or third party stimulator cells were assessed. In the experiment shown in Fig. 8, $CD4^+$ cells previously sensitized to the alloantigens of a given donor in the absence of TGF-β did not affect the generation of CTL activity by fresh T cells against donor B, as shown above. However, the addition of these control $CD4^+$ cells to T cells responding to third party, donor C stimulator cells increased the CTL activity generated. By contrast, CD4reg specific for donor B also suppressed the development of CTL activity against donor C.

Unlike the suppressive effects of the $CD4^+CD25^+$ T cells described by others which could be overcome by exogenous IL-2 or anti-CD28 (13, 14), the addition of IL-2 to CD4reg only partially blocked their suppressive effects. As expected, IL-2 augmented the generation of CTL activity by responder T cells. The addition of IL-2 to control $CD4^+$ cells restored CTL activity to baseline levels. However, the addition of IL-2 to cultures containing CD4reg did not completely restore CTL activity (Fig. 9). Thus, at least some of the responder T cells remained nonresponsive even in the presence of IL-2. This experiment has been repeated three times with similar results.

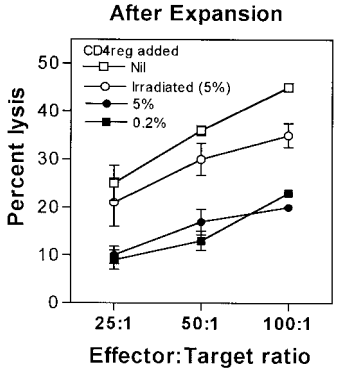

FIGURE 6. Expanded CD4reg retain their potent suppressive effects. The $CD4^+CD25^+$ fraction of naive $CD4^+$ cells primed in the presence of TGF-β was obtained by cell sorting and cultured in the presence of IL-2 (10 U/ml) for 5 days. The percentages indicated were added to fresh T cells and examined for inhibition of the generation of CTL activity. Irradiated $CD4^+CD25^+$ cells served as controls. This is one of two such experiments.

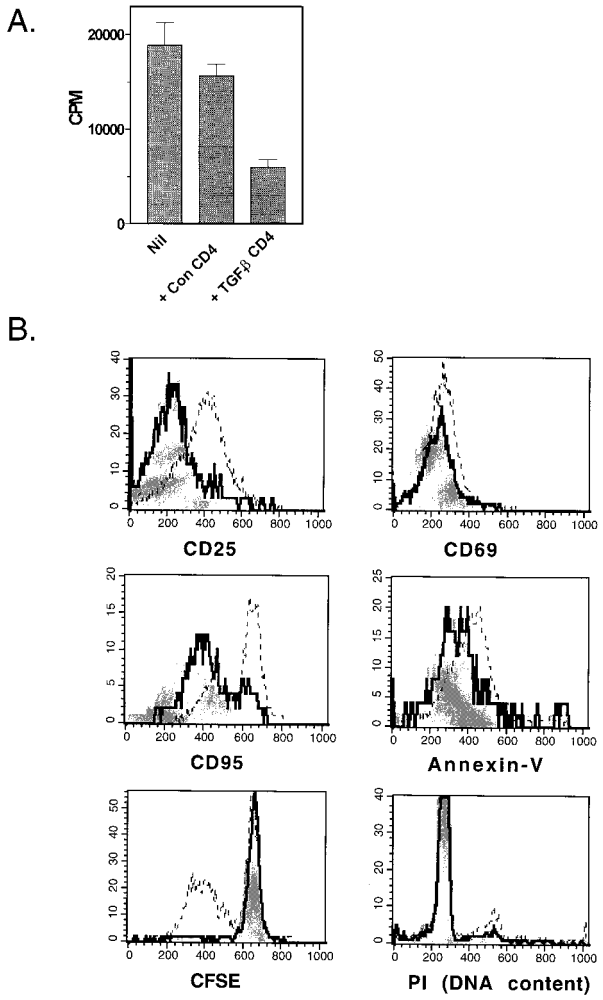

FIGURE 7. CD4reg block the proliferative response of responder T cells to alloantigens and $CD8^+$ T cells are their principal target. *A*, CD4reg or primed control (Con) $CD4^+$ cells were mixed with fresh T cells in a 1:4 ratio (10^5/well) and cultured in triplicate with irradiated stimulator cells (10^5/well) for 5 days. The cultures were pulsed with $[^3H]$TdR for the last 18 h. *B*, Phenotype of the cultured $CD8^+$ cells as determined by flow cytometry. Gating on $CD8^+$ cells, the gray area indicates cells cultured for 5 days without stimulator cells. Thick line, CD8 cells cultured with stimulator cells and CD4reg; thin dotted line, alloactivated CD8 cells cultured with control CD4 cells. The lymphocytes (10^5) were labeled with FITC-conjugated (anti-CD69), PE-conjugated (anti-CD25, anti-CD95) or Cy-Chrome-conjugated (anti-CD8) mAbs. Annexin V straining, CFSE levels, and DNA content by propidium iodide (PI) staining are also shown. These experiments were performed at least three times.

Because the naive fraction of $CD4^+$ cells contains only rare $CD25^+$ cells (Fig. 10*A*), we reasoned that TGF-β had costimulated $CD25^-$ T cells to develop suppressive activity. However, we could not exclude the possibility that these CD4reg were derived from the $CD25^+$ subset. To test this possibility, naive $CD4^+$ cells were depleted of $CD25^+$ cells and then primed in the presence of TGF-β. We found that, as before, TGF-β enhanced the expression of CD25 and the percentage of $CD25^+$ cells. Moreover, the percentage of $CD25^+$ cells after alloactivation was only modestly less in the subset that had initially been depleted of cells bearing this marker. However, after depletion of $CD25^+$ naive $CD4^+$ cells, the suppressive activity induced by TGF-β was markedly reduced

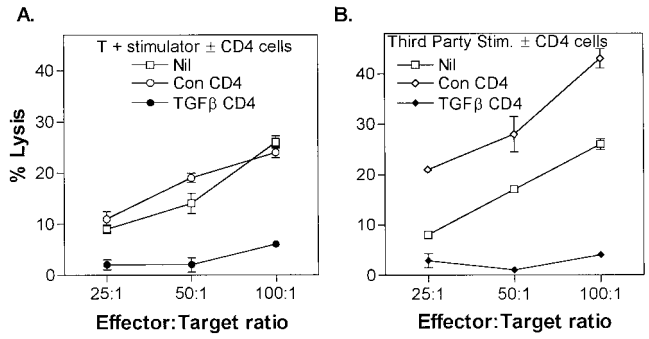

FIGURE 8. The suppressive effects of CD4reg are Ag nonspecific. The experimental protocol is similar to the studies described above. CD4reg cells from donor A displaying the activated phenotype shown in Fig. 3 after 5 days of culture, were added to autologous T cells and allogeneic stimulator cells from donor B (*A*) or donor C (*B*) and cultured for an additional 5 days. CTL activity against Con A blasts from each of the allogeneic donors is shown. This experiment has been performed three times. Con, Control.

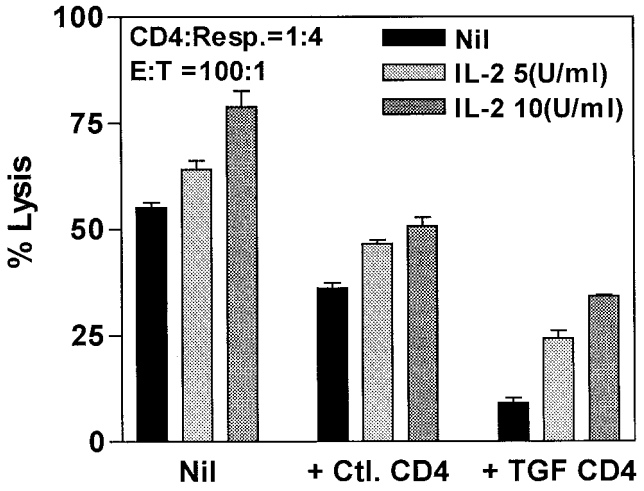

FIGURE 9. The suppressive activity of CD4reg on CTL activity was only partially abrogated by the addition of IL-2. T cells were alloactivated for 5 days with irradiated stimulator cells in the presence or absence of IL-2 (10 or 50 U/ml). The effect of allo-primed CD4con or CD4reg on the generation of CTL activity is shown. Data are representative of four experiments. Resp., responder cells.

(Fig. 10*B*). This finding suggests that the regulatory T cells induced by TGF-β are the progeny of thymus-derived $CD4^+CD25^+$ T cells.

Discussion

In this study, we have found that the costimulatory effects of TGF-β on naive $CD4^+$ $CD45RA^+$ T cells circulating in the periphery can result in the development of remarkably potent suppressive activity. TGF-β enhanced expression of CD25 and CTLA-4 and accelerated their differentiation to the $CD45RA^-$-activated phenotype. The phenotype and suppressive effects of these $CD4^+$ cells were similar, if not identical, with the thymus-derived $CD4^+CD25^+$-regulatory T cells described by others (3–8, 33). Moreover, we have shown that CD4reg block $CD8^+$ T cells from responding to alloantigens and that this effect is only partially reversed by exogenous IL-2. As expected, suppressive activity was also found in the $CD45RA^-RO^+$ fraction of $CD4^+$cells, and TGF-β could also enhance this activity, but this effect was more variable.

Previously we had reported that TGF-β has a crucial role in the generation of $CD8^+$ T cells that suppress Ab production (29, 30). In those studies, $CD8^+$ cells were activated with Con A in the presence of TGF-β for 24 h, whereas in the present study alloantigen-activated $CD4^+$ cells required a longer exposure with this cytokine for optimal up-regulation of CD25 and induction of potent suppressive activity. The more vigorous response of alloactivated naive $CD4^+$ cells in the presence of TGF-β and the increased viability of these activated cells are consistent with the previously described positive effects of this cytokine on naive T cells (25, 26). We suggest that the costimulatory effects of TGF-β on naive T cells induce them to develop down-regulatory activity instead of becoming conventional T effector cells.

A surprising finding of this study was the principal source of CD4reg. Depletion studies revealed that these cells were derived from the rare $CD25^+$ cells present in the $CD45RA^+RO^-$ fraction of peripheral blood. As previously reported by others, TGF-β can up-regulate CD25 expression by activated naive T cells (25). However, those that were $CD25^-$ before activation generally had minimal suppressive activity at the time they were examined. It is not unlikely, however, that naive $CD4^+CD25^-$ cells can also develop down-regulatory activity. We have recently observed that naive $CD4^+$ T cells primed with superantigen in the presence of TGF-β produce inhibitory amounts of active TGF-β when restimulated one or more times (34).

Consistent with studies of murine $CD4^+CD25^+$ regulatory T cells, human CD4reg induced by TGF-β inhibit T cell responses by blocking responsiveness rather than by killing them. Although we considered the possibility that the failure of $CD8^+$ cells to develop CTL activity could be due to cytolytic effects of CD4reg against the allogeneic stimulator cells, this was unlikely for the following reasons: 1) the numbers of CD4reg required for significant inhibition were too small for a significant effect on the Ag-presenting cells; 2) CD4reg had weak CTL activity, even after expansion in IL-2; and 3) other investigators have reported that TGF-β inhibits the development of CTL activity (21, 22).

Murine $CD4^+CD25^+$ cells inhibit IL-2 production by Ag-stimulated T cells and thereby block T cell proliferation (14, 15), a result in agreement with the present study. Another group has reported that $CD4^+CD25^+$ cells render $CD8^+$ cells anergic. These workers injected semiallogeneic splenocytes into neonatal mice and observed long term Ag-specific CD8 cell nonresponsiveness. Interestingly, although IL-2 could not restore CD8 CTL activity, this result was achieved after removal of regulatory $CD4^+CD25^+$ T cells from the cell suspensions (18). Thus, as long as regulatory T cells were present, even IL-2 could not reverse anergy. In in vitro studies, the anergy induced by mouse $CD4^+CD25^+$ cells can be overcome by IL-2 or anti-CD28 (14, 15). In the present experiments, the addition of exogenous IL-2 to cultures containing human CD4reg restored CD8 CTL activity partially but not completely. This result is consistent with the role of $CD4^+CD25^+$ cells in sustaining transplantation tolerance (18).

The suppressive effects of human CD4reg, like mouse $CD4^+CD25^+$ T cells, are Ag nonspecific. Once activated, these regulatory T cells have broad suppressive effects (14, 15). In the present experiments, besides inhibiting T cells responding to the same stimulator cells used to induce them, human CD4reg equally inhibited T cells responding to third party stimulator cells.

CTLA-4 is a potent inhibitor of T cell responses (35, 36). Two groups have recently reported that CTLA-4 plays a key role in the T cell-mediated dominant immunological self tolerance. Blockade of CTLA-4 abolished the suppressive activities of $CD4^+CD25^+$ T

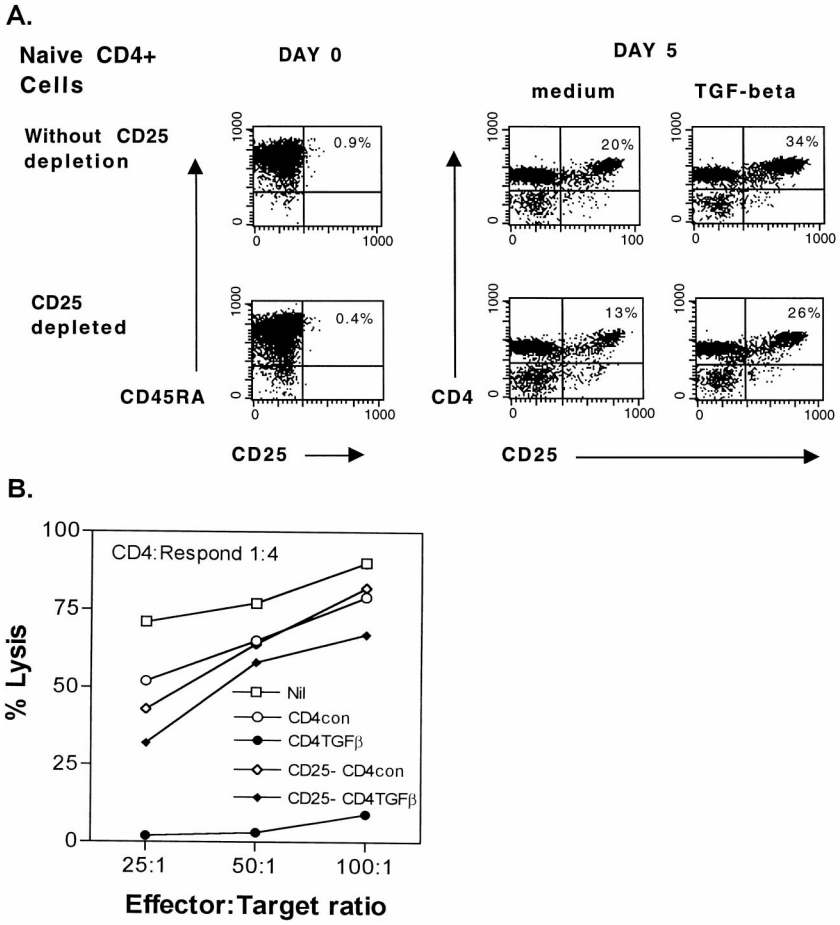

FIGURE 10. Depletion of $CD25^+$ cells from the starting population impairs the generation of regulatory cells. Naive $CD4^+$ cells were prepared with or without anti-CD25 Abs included in the staining cocktail to deplete the $CD25^+$ subset which comprised <1%. These cells were cultured with irradiated allogeneic stimulator cells ± TGF-β (0.1 ng/ml). After 5 days, the cells were assayed for CD25 expression (*A*) or for regulatory activity (*B*) as described above at a CD4reg-T cell ratio of 1:4 and examined for inhibition of the generation of CTL activity.

cells (37, 38). In this study, the costimulatory effects of TGF-β markedly up-regulated CTLA-4. In preliminary studies, Fab anti-CTLA-4 Abs obtained from Dr. B. M. Carreno (Genetics Institute, Cambridge, MA) did not inhibit the suppressive effects of CD4reg.

The mechanism of action of CD4reg remains to be determined but is clearly different from Tr1 $CD4^+$ cells repeatedly stimulated with IL-10 or activated with immature dendritic cells (32, 39). Tr1 cells are anergic, and their immunosuppressive effects are mediated by IL-10 and TGF-β. Anergic T cells suppress other T cell responses by targeting APC (40, 41). Under the experimental conditions described here, IL-10 could not be substituted for TGF-β to induce CD4reg (Fig. 1*B*). Moreover, CD4reg had the characteristics of activated rather than anergic cells in primary cultures (Fig. 3), and on restimulation with alloantigen these T cells proliferated even more vigorously than control $CD4^+$ cells (Fig. 5). In agreement with the studies of Thornton and Shevach (15), the suppressive activities were abolished by separation of the regulatory cells and effector cells by a semipermeable membrane, whereas neutralizing anti-TGF-β and anti-IL-10 Abs did not have this effect (Fig. 2*C*).

To explain how the addition of <1 regulatory T cell to 100 T cells can have potent inhibitory effects, it is likely that these cells expand rapidly. Consistent with this possibility, the suppressive effects of CD4reg were radiosensitive even after they were generated (Fig. 6). The effects of irradiation on suppressor T cells are well known (42–45). It is also possible that CD4reg are part of a network that facilitates other cells to produce inhibitory cytokines (46). This would explain why CD4reg have contact-dependent inhibitory effects in vitro but appear to depend on the production of TGF-β and IL-10 in vivo (2, 47, 48).

The ability to generate powerful regulatory T cells ex vivo has immediate clinical relevance. Allogeneic stem cell transplantation has great potential for the treatment of certain individuals with neoplastic or heritable diseases, but is limited by graft vs host disease. Similarly, although there has been great progress in managing acute rejection after transplantation of solid organs, survival of kidney grafts after 1 year has not increased significantly (49). The use of regulatory T cells generated ex vivo for the prevention of graft rejection as well as a treatment for autoimmunity has great potential. Unlike Tr1-like cells, both murine $CD4^+CD25^+$ T cells (15) and the comparable human T cell subset described in this report can be propagated ex vivo and retain their powerful suppressive effects after expansion. The positive effects of TGF-β on the viability of these regulatory cells are also encouraging. These findings add to the potential of adoptive regulatory T cell immunotherapy as a novel treatment strategy that will lack the toxic side effects of the agents currently in use.

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