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TGF-β Requires CTLA-4 Early after T Cell Activation to Induce FoxP3 and Generate Adaptive CD4$^+$CD25$^+$ Regulatory Cells[1]

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Although positive CD28 costimulation is needed for the generation of natural CD4$^+$CD25$^+$ regulatory T cells, we report that negative CTLA-4 costimulation is necessary for generating phenotypically and functionally similar adaptive CD4$^+$CD25$^+$ suppressor cells. TGF-β could not induce CD4$^+$CD25$^-$ cells from CTLA-4$^{-/-}$ mice to express normal levels of FoxP3 or to develop suppressor activity. Moreover, blockade of CTLA-4 following activation of wild-type CD4$^+$ cells abolished the ability of TGF-β to induce FoxP3-expressing mouse suppressor cells. TGF-β accelerated expression of CTLA-4, and time course studies suggested that CTLA-4 ligation of CD80 shortly after T cell activation enables TGF-β to induce CD4$^+$CD25$^-$ cells to express FoxP3 and develop suppressor activity. TGF-β also enhanced CD4$^+$ cell expression of CD80. Thus, CTLA-4 has an essential role in the generation of acquired CD4$^+$CD25$^+$ suppressor cells in addition to its other inhibitory effects. Although natural CD4$^+$CD25$^+$ cells develop normally in CTLA-4$^{-/-}$ mice, the lack of TGF-β-induced, peripheral CD4$^+$CD25$^+$ suppressor cells in these mice may contribute to their rapid demise. *The Journal of Immunology,* **2006, 176: 3321–3329.**

The CD4$^+$CD25$^+$ regulatory/suppressor T cells (CD4regs) are essential for tolerance to self Ags, for immunologic homeostasis and can also prevent allograft rejection (1, 2). CD4regs, however, are heterogeneous and can be divided into subsets that develop naturally in the thymus or are acquired peripherally (3). Following our observations with CD8$^+$ cells (4), we reported that the combination of IL-2 and TGF-β induces naive or total CD4$^+$CD25$^-$ cells to develop strong suppressive effects both in vitro and in vivo (5–7). Others have also reported that TGF-β can induce activated CD4$^+$ cells to become suppressor cells with a phenotype and functional profile similar to natural CD4$^+$CD25$^+$ regulatory cells (8–10). Both natural and acquired CD4regs express FoxP3, the transcription factor that is responsible for their development (8, 11, 12). Although it is known that CD28/B7 costimulation is required for the development of natural CD4$^+$CD25$^+$ cells (13, 14), the costimulatory requirements for the generation of TGF-β-induced CD4regs have not been defined. CTLA-4 and CD28 are homologs that each bind B7, but have opposing functions. Although CD28 costimulates T cells, CTLA-4 inhibits T cell activation by blocking cytokine production and cell cycle progression (15, 16). CTLA-4 is rapidly induced after T cell activation and has striking inhibitory properties (17). Delivery of anti-CTLA-4 mAbs can abrogate the induction of Ag-specific tolerance, enhance tumor responses, and markedly enhance autoimmune diseases (17). CTLA-4 is essential for maintaining immune homeostasis because genetically deficient mice develop a fatal lymphoproliferative disease with multiple organ pathology shortly after birth (18). This disease is T cell-mediated autoimmune because it is dependent upon costimulation and MHC class II (19).

Several mechanisms have been proposed to account for the inhibitory effects of CTLA-4: 1) because its affinity for B7 is greater than CD28, CTLA-4 may compete successfully for B7 binding sites on the Ag-presenting cell (20, 21); 2) CTLA-4 induces tryptophan catabolic products that inhibit T cell activation (22, 23); 3) cross-linking cell surface CTLA-4 may induce TGF-β production (24), although this is controversial (25); 4) intracellular CTLA-4 can localize in the immunological synapse and interfere with TCR signaling (26, 27); and 5) CTLA-4 appears to have a role in the function of T regulatory (Treg)[3] cells (28–31), and one group suggested a possible role in the generation of these cells (31).

Similar to CTLA-4-deficient mice (18), TGF-β and FoxP3-deficient (FoxP3$^{-/-}$) mice all develop the fatal lymphoproliferative syndrome indicated earlier (32, 33). Interestingly, forced expression of FoxP3 in CTLA-4$^{-/-}$ mice delays the disease (34). Because TGF-β induces expression of FoxP3 and up-regulates CTLA-4 expression, we asked whether CTLA-4 might be a critical intermediate in this phenomenon. Studies in CTLA-4-deficient mice have revealed that this is the case. Further studies with wild-type CD4$^+$CD25$^-$ cells provided additional support for the hypothesis that CTLA-4 has an essential role in the development of TGF-β-induced CD4regs. In accessory cell-dependent T cell activation, our studies suggest that CTLA-4 ligation of CD80 is required for TGF-β to induce FoxP3 and generate suppressor T cells.

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[3] Abbreviations used in this paper: HPRT, hypoxanthine guanine phosphoribosyltransferase; Treg, T regulatory.

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Materials and Methods

Animals

C57BL/6 mice were purchased from The Jackson Laboratory. CTLA-4 knockout mice were maintained as CTLA-$4^{+/-}$ heterozygotes. CTLA-$4^{-/-}$ mice were generated by breeding male and female CTLA-$4^{+/-}$ mice and screening the offspring for the homozygous-deficient genotype (19). Approximately 25% of the offspring were deficient genotype and 25% were wild type and used as controls. TCR transgenic DO11.10 mice were a gift from Dr. S. Stohlman (University of Southern California, Los Angeles, CA). 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-CD11b (M1/70), anti-CD8 (53-6.7), anti-B220 (RA36B2), anti-CTLA-4 (UC10-4B9), anti-CD2 (PM2-5), anti-MHC class I (28-14-8), an anti-cyclin D1 kit (DCS-6), and respective matched control IgG Abs were obtained from BD Pharmingen. Conjugated anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD25 (PC61.5), anti-CD103 (2E7), anti-CD80 (16-10A1), anti-CD86 (GL1) Abs, and matched control IgG Abs were obtained from eBioscience. The goat anti-glucocorticoid-induced TNFR polyclonal Ab and control IgG were purchased from R&D Systems. Unconjugated anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CTLA-4 (9H10), or matched control IgG Abs (all from eBioscience) were used for the neutralization experiments. Recombinant IL-2 and TGF-β1 were purchased from R&D Systems. Anti-phospho-smad3 was a gift from Dr. E. Leof (Mayo Clinic College of Medicine, Rochester, MN). 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 μg/ml streptomycin, and 10 mM HEPES (all 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) was used for all other cultures.

Cell isolation and culture

T cells were prepared from lymph node and spleen cells by collecting nylon wool column nonadherent cells as previously described (7). $CD4^+$ 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%). $CD4^+CD25^-$ cells were stimulated either with soluble anti-CD3 (0.25 μg/ml) or OVA 323–339 peptide (0.2 μg/ml) in the presence of irradiated (30 Gy) nylon adherent APC, or with anti-CD3/CD28 coated beads with or without TGF-β (0.2–10 ng/ml) in AIM-V serum-free medium for various days. In some experiments, anti-CTLA-4 (4 μg/ml), anti-CD80 (10 μg/ml), anti-CD86 (10 μg/ml), or control IgG (4–10 μg/ml) was added to cultures. For studies of cyclin expression, $CD4^+CD25^-$ cells were stimulated with soluble anti-CD3 from 24 to

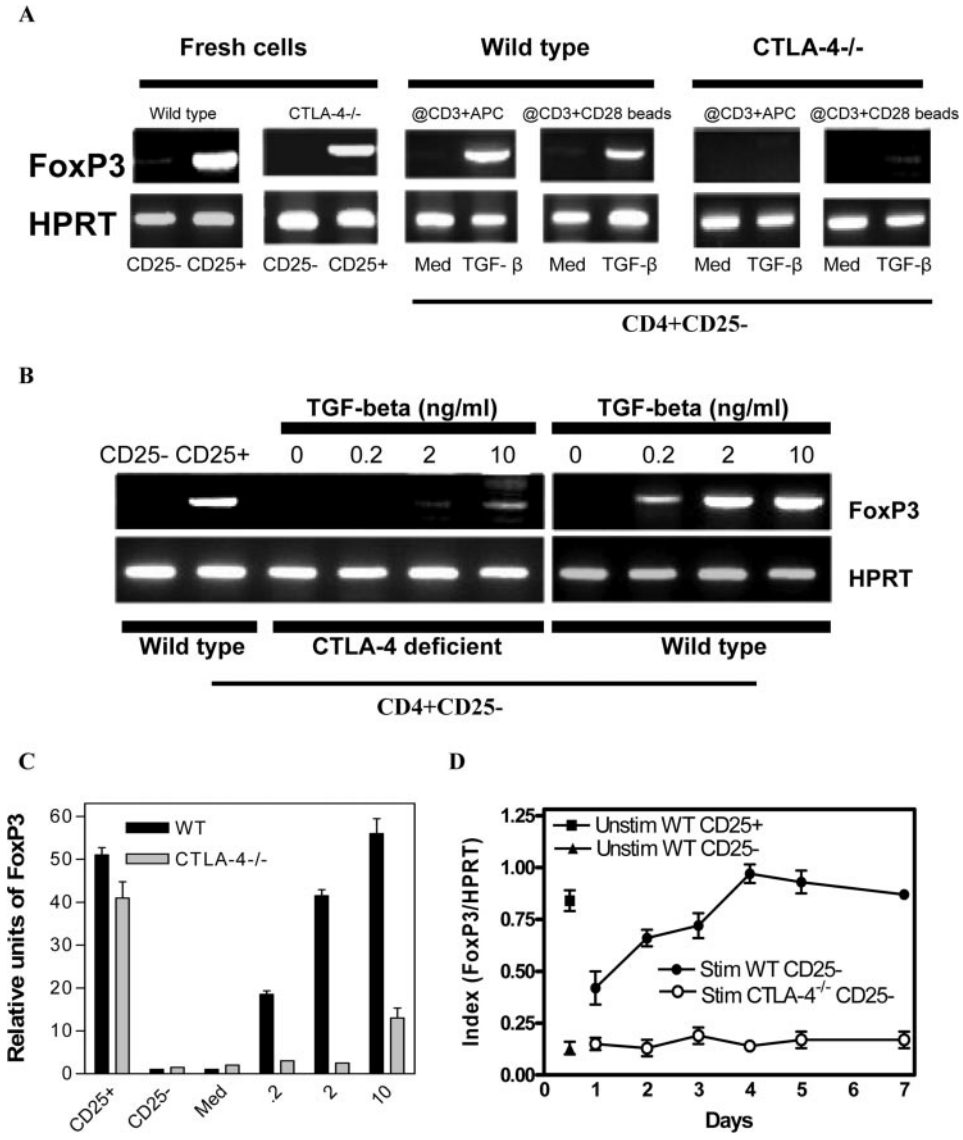

FIGURE 1. TGF-β induces the various activated $CD4^+CD25^-$ cells with dose-dependent effect to express FoxP3 in wild-type (WT) but not in CTLA-$4^{-/-}$ mice. *A*, Expression of FoxP3 mRNA by unstimulated $CD4^+CD25^+$ and $CD25^-$ cells from wild-type and CTLA-4-deficient mice was compared with $CD4^+CD25^-$ cells activated with soluble anti-CD3 (0.25 μg/ml) and APC, or anti-CD3 and anti-CD28 beads (1/20) with TGF-β1 (2 ng/ml) or without for 5–6 days. Purified $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from wild-type mice served as controls. The results shown are one of six independent experiments. *B* and *C*, $CD4^+CD25^-$ cells from wild-type and CTLA-$4^{-/-}$ mice were activated with soluble anti-CD3 and APC with various amounts of TGF-β1 for 5 days. FoxP3 mRNA was determined by semiquantitative and real-time PCR. This experiment is one of five independent experiments. *D*, $CD4^+CD25^-$ cells from wild-type and deficient mice were stimulated with soluble anti-CD3 and APC plus TGF-β1 for the days indicated, and FoxP3 mRNA was determined by RT-PCR. The levels of FoxP3 were analyzed by semiquantitative RT-PCR after normalization to HPRT. Each time point indicates the mean ± SEM of at least three experiments. Control $CD4^+CD25^-$ cells stimulated without TGF-β1 did not express FoxP3 at all times points indicated (data not shown).

48 h and stained for intracellular cyclin D according to instructions provided by the manufacturer.

Assessment of Treg cell activity in vitro

To generate Treg cells, $CD4^+CD25^-$ cells from either wild-type or $CTLA4^{-/-}$ mice were stimulated with anti-CD3 (0.25 μg/ml) in the presence of irradiated APC with TGF-β (2–10 ng/ml) ($CD4_{TGF\text{-}\beta}$) or without ($CD4_{med}$) for 5 days. Various doses of $CD4^+$ regulatory cells ($CD4_{TGF\text{-}\beta}$) or control $CD4^+$ cells ($CD4_{med}$) were added to fresh T cells that were activated with anti-CD3 (0.25 μg/ml) and in the presence of irradiated APC. Proliferation was assayed by [^{3}H]thymidine incorporation. In some experiments, responder T cells were labeled with CFSE as previously described (6), and inhibition of cycling T cells was assessed.

RT-PCR and real-time PCR

Total RNA was extracted from cells using TRIzol reagent and used to determine the expression and relative level of the transcription factor Foxp3 and CTLA-4. First strand cDNA was synthesized using Omniscript Reverse Transcriptase kit with random hexamer primers. FoxP3 and HPRT (hypoxanthine guanine phosphoribosyltransferase) mRNA was measured by semiquantitative RT-PCR using published primers (35). Real-time PCR was performed with a LightCycler, and message levels were quantified using the LightCycler Fast Start DNA Master Green I kit according to the manufacturer's instructions. The relative expression of FoxP3 and CTLA-4 was determined by normalizing expression of each target to β-actin using the primers: Foxp3, 5′-ACTGGGGTCTTCTCCCTCAA-3′, 5′-CGTGGGAAGGTGCAGAGTAG-3′; CTLA-4, 5′-GTTGGGGGCATTTTCACATA-3′, 5′-TTTTACAGTTTCCTGGTCTC-3′; and β-actin, 5′-TGACAGGATGCAGAAGGAGA-3′, 5′-GTACTTGCGCTCAGGAGGAG-3′.

Western blot analysis

A total of 5×10^6 cells from wild-type and $CTLA\text{-}4^{-/-}$ mice was incubated at 37°C for 30 min with TGF-β (0.2, 2 ng/ml) or without, lysed, and probed for expression of phospho-smad3 by Western blot analysis with an anti-phospho-smad3 polyclonal Ab provided by Dr. E. B. Leof. The loading was confirmed by anti-actin Ab (Sigma-Aldrich).

Statistical analysis

Statistical comparison between various groups was performed by the *t* test using GraphPad PRISM software (GraphPad).

Results

Although endogenous $CD4^+CD25^+$ cells from $CTLA\text{-}4^{-/-}$ mice express FoxP3 mRNA (31) (Fig. 1*A*), TGF-β had a markedly decreased ability to induce $CD4^+CD25^-$ cells from these animals to express this transcription factor. Previous workers have reported that $CD4^+CD25^+$ regulatory cells in $CTLA\text{-}4^{-/-}$ mice display suppressive activity (28, 31). Although Takahashi et al. (28) indicated that this activity was reduced, Tang and coworkers (31), using a mouse model that minimized activated $CD25^+$ cells, found that endogenous $CD4^+CD25^+$ cells from $CTLA\text{-}4^{-/-}$ mice expressed FoxP3 and that their suppressive activity was intact.

The different effects of TGF-β on $CD4^+CD25^-$ cells from $CTLA\text{-}4^{-/-}$ mice and wild-type mice on FoxP3 expression was striking whether T cells were stimulated by anti-CD3 with accessory cells or with accessory cell-independent anti-CD28 costimulation (Fig. 1*A*). TGF-β at 2 ng/ml was the optimal concentration needed for this cytokine to induce suppressor cells in wild-type mice (Fig. 2*A*). This amount of TGF-β failed to induce FoxP3 expression in $CTLA\text{-}4^{-/-}$ mice as measured by semiquantitative and real-time PCR (Fig. 1, *B* and *C*). Dose-response studies revealed that TGF-β at 10 ng/ml, five times the optimal concentration, only weakly induced FoxP3 in these mice (Fig. 1, *B* and *C*). Even this concentration of TGF-β was unable to induce $CD4^+CD25^-$ cells from $CTLA\text{-}4^{-/-}$ mice to develop suppressive activity (Fig. 2*B*). Time course studies revealed that TGF-β rapidly induced and sustained FoxP3 mRNA in wild-type mice, but $CD4^+CD25^-$ cells from deficient mice did not express FoxP3 at any time point tested (Fig. 1*D*).

The inability of TGF-β to induce FoxP3 could not be explained by a failure of T cells from $CTLA\text{-}4^{-/-}$ to respond to TGF-β. Following binding to specific receptors TGF-β phosphorylates various Smads to initiate its inhibitory effects. Fig. 3*A* shows that treatment of CTLA-4-deficient and wild-type $CD4^+CD25^-$ cells

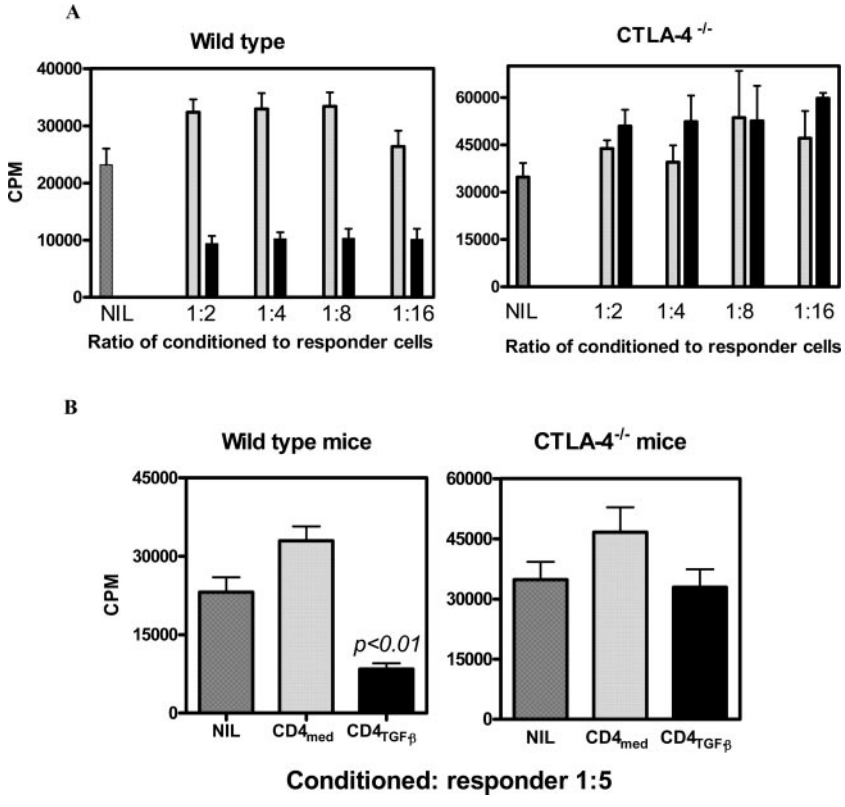

FIGURE 2. TGF-β induces $CD4^+CD25^-$ cells from wild-type but not from $CTLA\text{-}4^{-/-}$ mice to develop into $CD4^+$ Treg cells. *A*, $CD4^+CD25^-$ cells from either wild-type or $CTLA\text{-}4^{-/-}$ mice were stimulated with soluble anti-CD3 and APC with TGF-β1 ($CD4_{TGF\text{-}\beta}$) (2 ng/ml) or without ($CD4_{med}$) for 5 days. Suppression was assayed using anti-CD3-stimulated cells as described in *Materials and Methods*. The ratios of $CD4_{med}$ (▨) or $CD4_{TGF\text{-}\beta}$ (■) to responder T cells are shown. *B*, A similar design as in *A*, except 10 ng/ml TGF-β1 was used to condition $CD4^+CD25^-$ cells. Suppression was assessed at a 1:5 ratio. This result is representative of four independent experiments. NIL indicates no added cells.

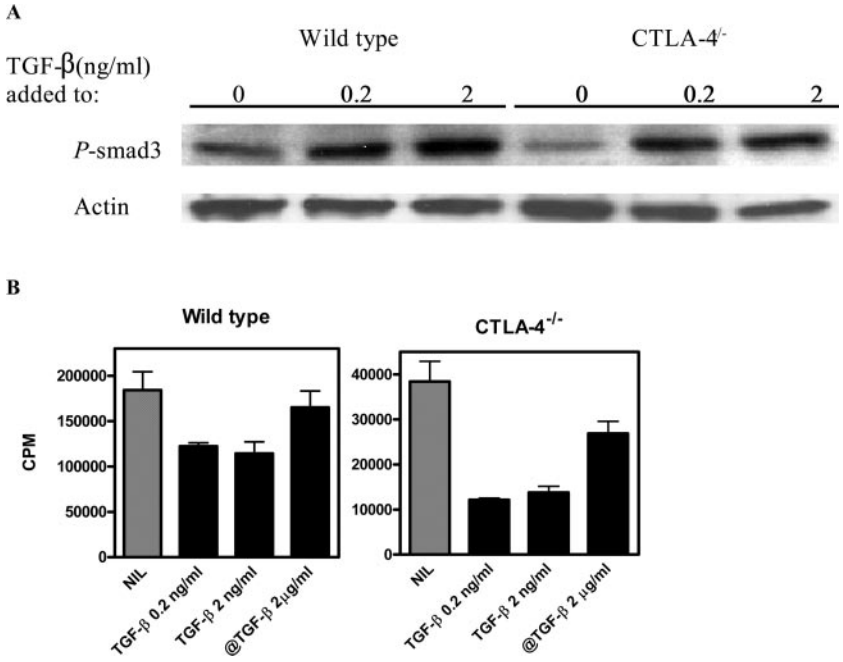

FIGURE 3. $CD4^+CD25^-$ cells from CTLA-$4^{-/-}$ mice respond to TGF-β. *A*, $CD4^+CD25^-$ cells from wild-type or CTLA-$4^{-/-}$ mice were incubated at 37°C with or without TGF-β1 (0.2–2 ng/ml) for 30 min. Protein levels of phospho-smad3 (*P*-smad3) or actin were determined by Western blot. This experiment is one representative of four independent experiments. *B*, $CD4^+CD25^-$ cells from wild-type or CTLA-$4^{-/-}$ mice were stimulated with anti-CD3 as in *A* with or without TGF-β (0.2–2 ng/ml) for 48 h. In some experiments anti-TGF-β (2 μg/ml) was added to TGF-β (2 ng/ml). Proliferation was assayed by [^{3}H]thymidine incorporation. NIL indicates no additives, medium only.

with TGF-β resulted in similar phosphorylation of Smad3. Moreover, various doses of TGF-β similarly inhibited the T cell proliferative response of both wild-type and CTLA-4-deficient mice to anti-CD3 (Fig. 3*B*).

We next considered whether CTLA-4 was needed for TGF-β to induce expression of FoxP3 in wild-type mice. As shown in Fig. 4, the addition of anti-CTLA-4 to $CD4^+CD25^-$ cells stimulated with anti-CD3 plus APC (Fig. 4*A*) or anti-CD3/28 beads (Fig. 4*B*), completely abolished the ability of TGF-β to induce FoxP3 mRNA. By contrast, expression of glucocorticoid-induced TNFR and CD103 was increased (data not shown). We also used anti-MHC class I and anti-CD2 Abs as controls to exclude the possibility that IgG binding to the T cell surface membrane can nonspecifically inhibit TGF-β-induced expression of FoxP3 (data not shown). In addition to polyclonally activated cells, we also documented that the addition of anti-CTLA-4 blocked FoxP3 expression by Ag-specific stimulation in the presence of TGF-β (Fig. 4*C*).

Anti-CTLA-4 also abolished the ability of TGF-β to induce suppressor cells. Fig. 5 shows that the suppressive activity of $CD4^+$ cells generated with anti-CTLA-4 was abolished as indicated by reduced numbers of total T cells (Fig. 5*A*) and cycling $CD8^+$ or $CD8^-$ cells (Fig. 5, *B* and *C*).

Next, we considered the mechanism of action of CTLA-4. This inhibitory element is generally expressed by T cells 2 to 3 days after activation (16). However, June and colleagues (36) have reported that CTLA-4 has early effects on the threshold of T cell activation before it is detectable by flow cytometry. Previously, we reported that TGF-β needed to be present at the onset of T cell activation to induce either $CD8^+$ or $CD4^+$ cells to develop suppressive effects (4, 6). Although we and others have reported that TGF-β enhances expression of CTLA-4 (5, 6, 8, 37, 38), we also considered that TGF-β may accelerate its appearance. Fig. 6 shows that, indeed, this effect is the case. TGF-β markedly increased expression of CTLA-4 mRNA during the first 3 h after T cell activation. Increased surface expression of CTLA-4 induced by TGF-β was not observed until 24 h of culture. As expected, therefore, anti-CTLA-4 maximally inhibited FoxP3 expression when added at the start of the cultures (Fig. 7). The inhibitory activity was no longer present if added 2 days after T cell activation. Thus, small amounts of intracellular CTLA-4 may have a considerable effect in the development of peripheral CD4regs.

We next asked whether CTLA-4 required B7 ligation for its effect on FoxP3 expression. Using soluble anti-CD3 and APC, studies with blocking Abs revealed that anti-CD80, but not anti-CD86, inhibited TGF-β-induced expression of FoxP3. Similar to anti-CTLA-4, the greatest inhibitory effect of CD80 was when the mAb was added at the start of the culture. The inhibitory effect of anti-CD80, however, was less than anti-CTLA-4 (Fig. 7). The greater effect of anti-CD80 than CD86 is consistent with other reports that these two homologs differentially modulate the suppressive activities of $CD4^+CD25^+$ Treg cells. In these studies CD80 was the preferential ligand for CTLA-4 (39, 40).

We indicated earlier that anti-CTLA-4 could block TGF-β-induced FoxP3 expression by $CD4^+$ cells stimulated without accessory cells. Another report has shown that activated $CD4^+$ cells can express B7 (41). Therefore, we assessed B7 expression by $CD4^+$ cells stimulated with anti-CD3/28 beads with or without TGF-β. Not only did we find CD80 expressed by stimulated $CD4^+CD25^-$ cells, but also we found that TGF-β significantly enhanced expression of CD80, but not CD86 (Fig. 8).

To learn the functional consequences of accelerated CTLA-4 expression by TGF-β, we determined the effect of antagonizing this molecule on cyclin D expression, intracellular proteins normally silent, but expressed by cycling T cells (Fig. 9). Following stimulation by soluble anti-CD3, TGF-β transiently inhibited cyclin D1 expression (Fig. 9, *A* and *B*). This suppressive effect was observed during the first 24 h of culture, and inhibition of [^{3}H]thymidine incorporation was also observed ($p = 0.01$). The addition of anti-CTLA-4 blocked both the inhibitory effects of TGF-β on cyclin D expression and partially blocked inhibition of T cell proliferation (Fig. 9*C*). Thus, early inhibition of TGF-β on T cell proliferation reflects the combined effects of TGF-β and CTLA-4 induced by this cytokine. By 48 h of culture TGF-β inhibition of cyclin D was no longer present (Fig. 9*B*). In fact, costimulatory effects of TGF-β become evident after this time (5, 6).

Discussion

The principal findings of this study are that TGF-β accelerates the expression of CTLA-4 by stimulated $CD4^+CD25^-$ T cells, and that like TGF-β, CTLA-4 is needed early after T cell activation for

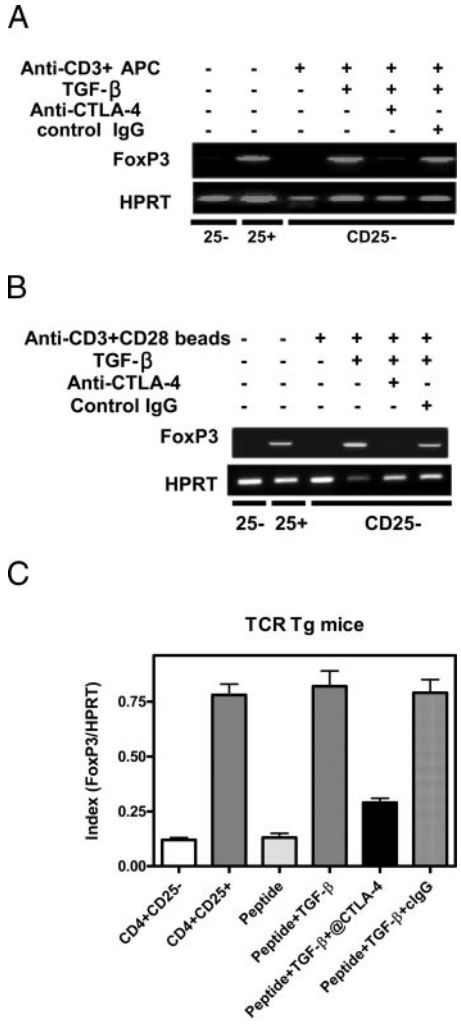

FIGURE 4. Abs to CTLA-4 block the induction of FoxP3 expression on activated $CD4^+CD25^-$ cells in wild-type mice. *A*, $CD4^+CD25^-$ cells from wild-type mice were stimulated with soluble anti-CD3 with TGF-β1 or without as described Fig. 2. Anti-CTLA-4 Ab (4 μg/ml) or control IgG (4 μg/ml) was added to some TGF-β cultured wells. *B*, These cells were also stimulated with anti-CD3 and anti-CD28 beads (1/20) with TGF-β1 (2 ng/ml) or without for 5 days, with anti-CTLA-4 Ab added as in *A*. FoxP3 mRNA was determined by RT-PCR. This result is representative of four independent experiments. *C*, $CD4^+CD25^-$ cells from DO11.10 transgenic (TCR Tg) mice were stimulated with OVA 323–339 peptide (0.2 μg/ml) with TGF-β1 (2 ng/ml) or without for 5 days, with anti-CTLA-4 Ab added as in *A*. The levels of FoxP3 were analyzed by semiquantitative RT-PCR after normalization to HPRT.

TGF-β to induce these cells to become $CD4^+CD25^+FoxP3^+$ suppressor cells. Blocking the activity of CTLA-4 during the first 24 h following polyclonal or Ag-specific T cell stimulation also blocked the ability of TGF-β to induce FoxP3. Previously, we reported that TGF-β must be present during the first 24 h following activation to induce $CD8^+$ or $CD4^+$ cells to become Treg cells (4, 6)

Our observations are consistent with other reports suggesting that T cell differentiation is determined by the duration and quality of the activating signals (42, 43). T cells respond to antigenic challenge when the number of TCR triggered reached an appropriate threshold (43). To become effector cells, T cells require strong, persistent stimulation. Mature APC bearing B7 and other costimulatory molecules provide stable, long-lasting TCR contacts that permit this differentiation. Immature APC, however, lack sufficient costimulatory molecules, and their brief contacts with T cells drive them to become anergic, a characteristic feature of Treg cells (44). CTLA-4 can destabilize CD28 binding the immunological synapse with APC (27) and interfere with T cell activation by B7-dependent or -independent effects (45). The relatively greater effects of CD80 in comparison with CD86 in enhancing TGF-β-induced FoxP3 expression is consistent with the greater binding affinity of CD80 for CTLA-4 (27, 39, 40). Thus, the combined inhibitory effects of TGF-β and CTLA-4 may convert strong, stable activating signals to brief unstable signals that favor anergy, expression of FoxP3, and Treg differentiation. To our knowledge, this is the first report that TGF-β can enhance T cell expression of CD80.

Another group has recently reported a role for CTLA-4 in the generation of alloantigen-specific $CD4^+CD25^+$ Treg cells. The transfer of allogeneic cells coated with anti-CTLA-4 into intact mice resulted in increased numbers of $CD4^+CD25^+$ cells expressing CTLA-4 (46). Others have also reported that CTLA-4 engagement can up-regulate IL-10 and TGF-β (24, 47). Finally, FoxP3 has been reported to up-regulate CTLA-4 expression (48). Thus a TGF-β/CTLA-4/FoxP3/CTLA-4 positive loop may be vital for the generation and maintenance of acquired $CD4^+CD25^+$ Treg cells. This relationship may explain why TGF-β1-deficient, CTLA-4-deficient, and FoxP3-deficient mice all develop a similar, rapidly fatal T cell-mediated, autoimmune lymphoproliferative disease shortly after birth (18, 32–34). Although natural $CD4^+CD25^+$ cells develop in the thymus of CTLA-$4^{-/-}$ mice (31), these cells, by themselves, cannot prevent this overwhelming autoimmune disease.

To learn how signals from CTLA-4 synergize with signals from TGF-β to induce FoxP3 expression, we determined the effect of blocking CTLA-4 on the ability of TGF-β to inhibit T cell function. Evidence was obtained that blocking CTLA-4 markedly decreased the ability of TGF-β to inhibit early cyclin D expression and T cell proliferation. Thus, the combined inhibitory activities of TGF-β and CTLA-4 induced by this cytokine may decrease T cell activating signals to levels that induce FoxP3 instead of the transcription factors responsible for Th cell differentiation (49).

TGF-β signaling involves Smad-dependent and -independent pathways (50) and the signaling pathways responsible for up-regulation of CTLA-4 remain to be elucidated. It is known, however, that TGF-β-activated Smads result in expression of c-*fos* (51), and it is possible that Smad-AP-1 interactions may affect CTLA-4 expression. The CTLA-4 promoter contains an AP-1 binding site (14). It has also been reported that both TGF-β and cAMP can increase CD40L expression, and a relationship between TGF-β and protein kinase A has been reported (52, 53). Thus, both Smad-dependent and -independent signaling pathways may be involved.

Other elements besides TGF-β appear to up-regulate FoxP3 expression. Recently, it has been reported that PGE_2 has this property (54). This agent up-regulates cAMP, and this element also can up-regulate CTLA-4 (55). Another group has reported that immature APCs whose maturation was blocked by a proteosome inhibitor also induce $CD4^+$ cells to express FoxP3 and develop suppressive effects (56). In each of these studies, expression of CD25 was not enhanced. By contrast, TGF-β up-regulates FoxP3 and enhances IL-2 signaling by up-regulating CD25 and CD122, the α- and β-chain of the IL-2R (5, 6, 12, 38, 57). Because of the importance of IL-2 in the development of Treg activity (58, 59), the combined effects of TGF-β on FoxP3 expression and enhanced IL-2 signaling probably explain the potent in vivo effects of CD4regs induced with TGF-β (S. G. Zheng, L. Meng, J. H. Wang, M. Watanabe, M. L. Barr, D. V. Cramer, J. D. Gray, and D. A. Horwitz, submitted for publication) (7, 8, 63). We have found that

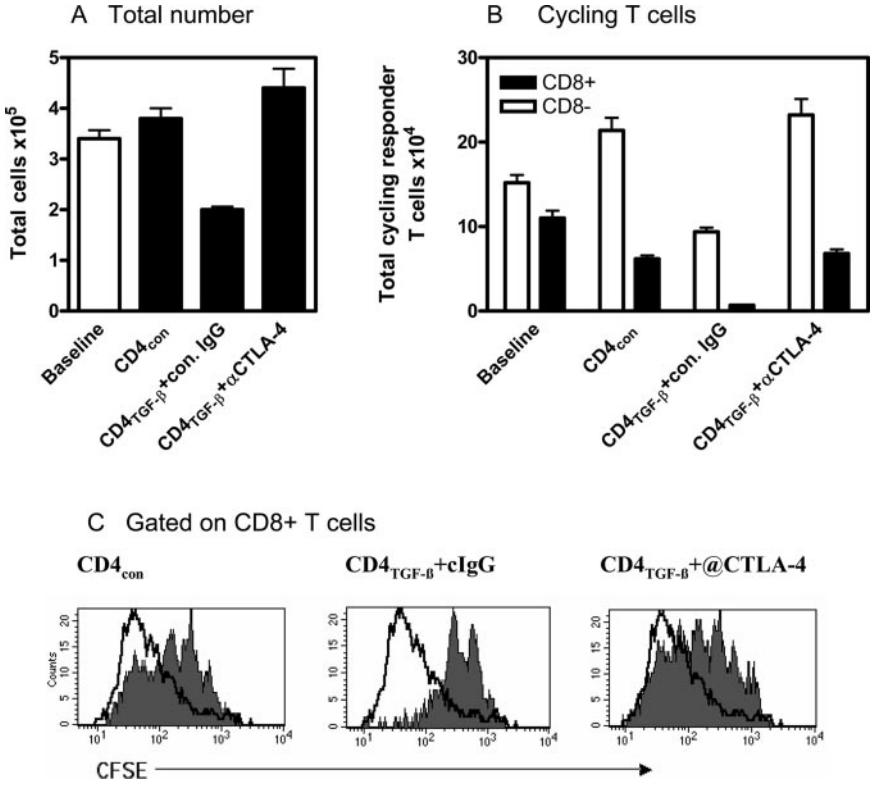

FIGURE 5. Abs to CTLA-4 block the ability of TGF-β to induce $CD4^+CD25^-$ cells from wild-type mice to develop suppressive activity. Using the protocol described in Figs. 2 and 4, $CD4_{med}$ and $CD4_{TGF\text{-}\beta}$ cells generated with anti-CTLA-4 or control IgG were added to 2×10^5 responder T cells labeled with CFSE in various ratios, stimulated with soluble anti-CD3 with APC (1×10^5), and cultured for 3 days. The ratio of conditioned CD4 to responder cells in the experiment shown was 1:4. *A*, Cell count of total cells. *B*, Total number of cycling cells (percentage of CFSE-positive cells that had divided $\times$ the total number). *C*, FACS profile $CD8^+$ responder cells. The cells were gated on $CD8^+$ cells and assessed for CFSE intensity. Shown are the proliferative response of the responder $CD8^+$ cells (solid line histogram) and the effect (shaded histogram) of adding $CD4_{con}$ (*left*), $CD4_{TGF\text{-}\beta}$ that had been generated with control IgG (*middle*), or with anti-CTLA-4 mAb (*right*). The result shown is representative of four experiments.

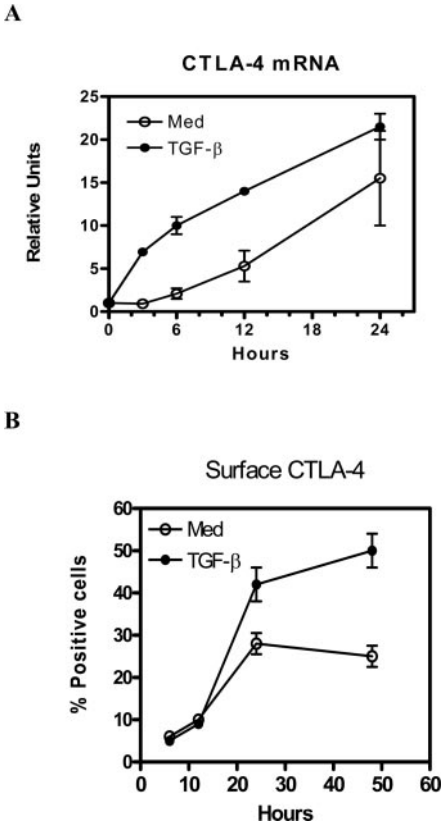

FIGURE 6. TGF-β accelerates CTLA-4 expression by $CD4^+$ cells. *A*, B6 $CD4^+CD25^-$ cells were stimulated with soluble anti-CD3 with TGF-β1 (2 ng/ml) or without for the hours indicated and CTLA-4 mRNA was determined by real-time PCR. Mean $\pm$ SEM is determined in relative units of triplicate samples and indicates level of CTLA-4 compared with β-actin. The result shown is one of two experiments. *B*, Surface expression of CTLA-4 of $CD4^+CD25^-$ cells stimulated with soluble anti-CD3 with or without TGF-β as determined by flow cytometry.

IL-2 markedly enhances TGF-β-induced FoxP3 expression (our unpublished observation).

Our previous studies suggested that in addition to their suppressive properties, natural CD4regs facilitate the generation of acquired CD4regs (12). We demonstrated that the positive effects of IL-2 and TGF-β on naive $CD4^+CD25^+$ cells enabled them to markedly increase the numbers and potency of $CD4^+CD25^+$ cells induced ex vivo by TGF-β from $CD4^+CD25^-$ precursors. Thus, it is probably the combined activities of TGF-β on both natural and acquired $CD4^+CD25^+$ cells that maintain normal immunologic homeostasis.

Therapeutic antagonism of CD28 with CTLA-4 Ig has been used to treat patients with rheumatoid arthritis (60), and has been

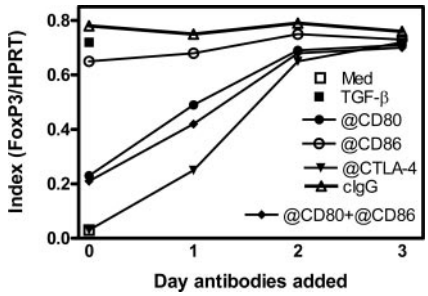

FIGURE 7. CTLA-4 and CD80 are required at the onset of T cell activation for maximal expression of TGF-β-induced FoxP3. $CD4^+CD25^-$ cells were stimulated with soluble anti-CD3 with or without TGF-β1 as described. Various blocking Abs or control IgG were added to cultures containing TGF-β at the days indicated. FoxP3 mRNA was determined by a semiquantitative RT-PCR. The result is representative of three separate experiments.

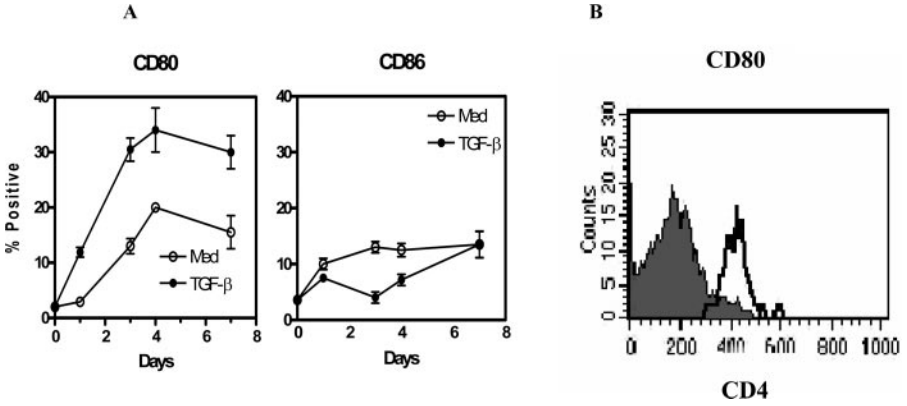

FIGURE 8. TGF-β enhances expression of CD80, but not CD86 by activated $CD4^+$ cells. *A*, Time course study of $CD4^+CD25^-$ cells stimulated with anti-CD3/28 beads with or without TGF-β1. Values shown indicate mean ± SEM of three separate experiments. *B*, Representative histogram of CD80 expression by $CD4^+$ cells stimulated for 5 days in medium (shaded histogram) with TGF-β (thick line histogram).

considered to sustain allograft survival (61). This agent, however, will also block CTLA-4 ligation to B7. Because CTLA-4 has an important role in the maintenance of immunologic tolerance (62), one consequence of therapeutic blockage of B7 might be to block the generation of adaptive $CD4^+CD25^+$ regulatory cells. Thus, this blockade may have unfortunate long-term consequences in patients who have autoimmune diseases or who have received organ allografts.

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Disclosures

The authors have no financial conflict of interest.

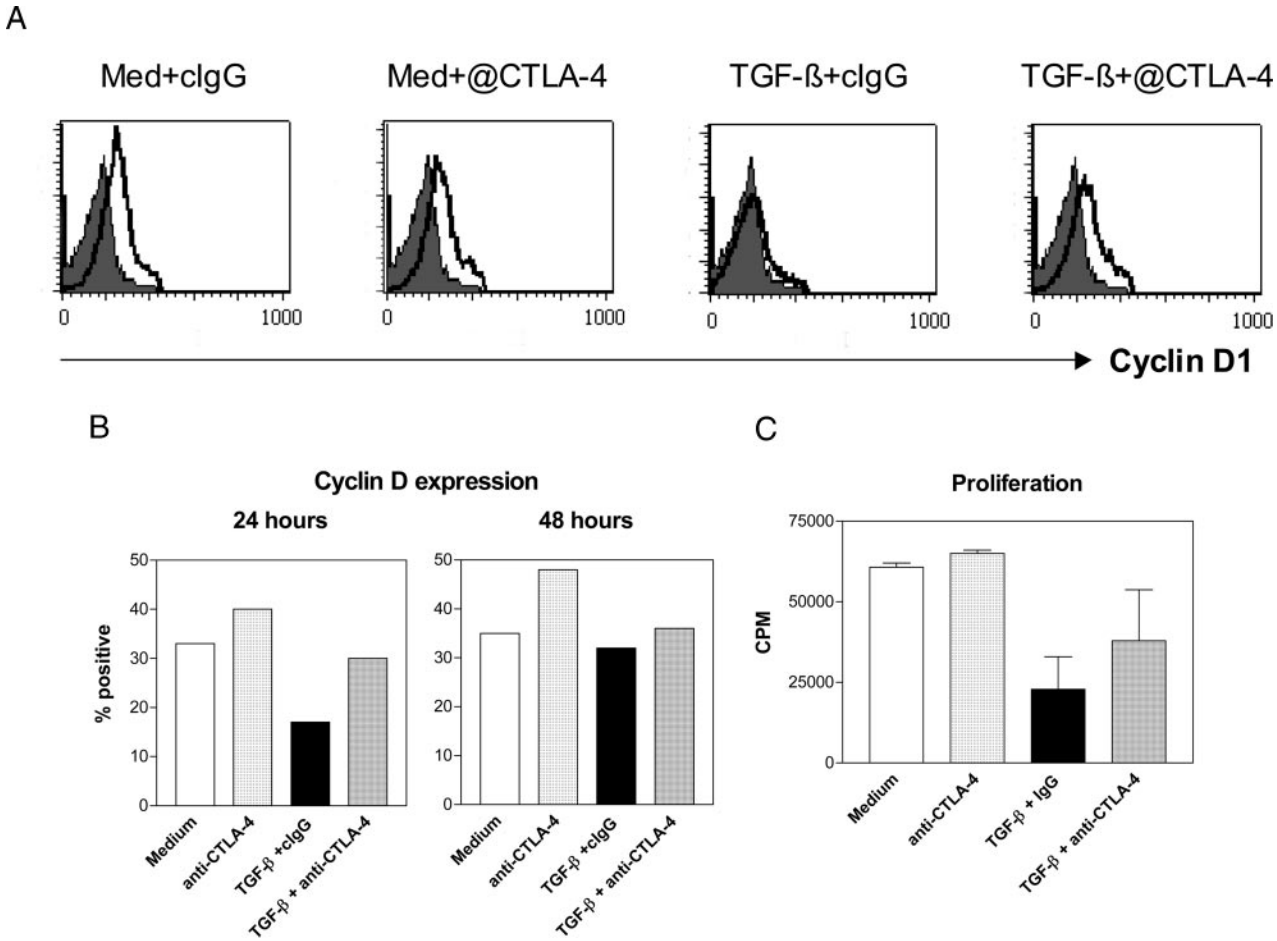

FIGURE 9. CTLA-4 up-regulated by TGF-β contributes to its early inhibitory effects. *A*, $CD4^+CD25^-$ cells were stimulated with soluble anti-CD3 with TGF-β1 or without for 24 h, and intracellular staining of cyclin D was determined by flow cytometry. Cells stained with control IgG (shaded histogram) and cells stained with anti-cyclin D1 (solid line histogram) are indicated. This experiment is representative of five separate experiments. *B*, Time course study of $CD4^+CD25^-$ cells stimulated for 24 and 48 h with the additives shown. *C*, $CD4^+CD25^-$ cells, as shown in *B*, were stimulated for 36 h in triplicate and [^{3}H]thymidine was added for the last 16 h. The result is representative of three separate experiments.

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