Engagement of Cytotoxic T Lymphocyte–associated Antigen 4 (CTLA-4) Induces Transforming Growth Factor β (TGF-β) Production by Murine CD4$^+$ T Cells

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Summary

Evidence indicates that cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) may negatively regulate T cell activation, but the basis for the inhibitory effect remains unknown. We report here that cross-linking of CTLA-4 induces transforming growth factor β (TGF-β) production by murine CD4$^+$ T cells. CD4$^+$ T helper type 1 (Th1), Th2, and Th0 clones all secrete TGF-β after antibody cross-linking of CTLA-4, indicating that induction of TGF-β by CTLA-4 signaling represents a ubiquitous feature of murine CD4$^+$ T cells. Stimulation of the CD3–T cell antigen receptor complex does not independently induce TGF-β, but is required for optimal CTLA-4–mediated TGF-β production. The consequences of cross-linking of CTLA-4, together with CD3 and CD28, include inhibition of T cell proliferation and interleukin (IL)-2 secretion, as well as suppression of both interferon γ (Th1) and IL-4 (Th2). Moreover, addition of anti–TGF-β partially reverses this T cell suppression. When CTLA-4 was cross-linked in T cell populations from TGF-β1 gene–deleted (TGF-β1$^{-/-}$) mice, the T cell responses were only suppressed 38% compared with 95% in wild-type mice. Our data demonstrate that engagement of CTLA-4 leads to CD4$^+$ T cell production of TGF-β, which, in part, contributes to the downregulation of T cell activation. CTLA-4, through TGF-β, may serve as a counterbalance for CD28 costimulation of IL-2 and CD4$^+$ T cell activation.

Key words: CD4$^+$ T cells • cytotoxic T lymphocyte–associated antigen 4 • transforming growth factor β

Recent evidence indicates that CTL-associated antigen 4 (CTLA-4),[1] a counterreceptor in addition to CD28 for the B7 family of costimulatory molecules, is a negative regulator of T cell activation (1–4), although this remains controversial (5). In vitro, antibody cross-linking of CTLA-4 has been shown to inhibit T cell proliferation and IL-2 production triggered by anti-CD3 and anti-CD28 antibodies, whereas soluble intact or Fab fragments of anti–CTLA-4 antibody enhance the proliferative response (2, 6–8). In vivo, blockade of CTLA-4 with soluble intact or Fab antibody fragments greatly enhances the T cell response to peptide antigen (9) or superantigen Staphylococcus enterotoxin B (10). Furthermore, in vivo administration of antibodies to CTLA-4 promotes tumor rejection (11) and exacerbates disease in experimental autoimmune encephalomyelitis (12). Direct evidence of a critical regulatory role for CTLA-4 comes from CTLA-4–deficient (−/−) mice, which manifest a severe T cell proliferative disorder with multiorgan lymphocytic infiltration and tissue destruction. The CTLA-4$^{-/-}$ animals die by 3–4 wk of age (13, 14). These results indicate that CTLA-4 is predominantly a negative regulator of T cell responses. However, the basis for the inhibitory effects mediated by CTLA-4 signaling has not yet been well elucidated. One report demonstrated that CTLA-4 mediates antigen-specific apoptosis of human T cells (15), whereas others indicate that the primary effect of CTLA-4 ligation is not the induction of apoptosis, at least in murine CD4$^+$ T cells (2, 8). In addition, T cells in CTLA-4$^{-/-}$ mice exhibit normal levels of apoptosis (16).

TGF-β elicits diverse cellular responses depending on cell type, state of differentiation, culture conditions, and presence of other cytokines (17–19). TGF-β is a potent inhibitor of the T cell–mediated immune response both in vitro (20, 21) and in vivo (22–25), although it can also exert upregulatory effects on T cell proliferative responses under some conditions (26–28). Interestingly, mice deficient in the TGF-β1 gene also develop massive lymphoproliferative disorders that lead to organ failure, and die at the age of 2–3 wk (29–31), similar to the pathogenesis in CTLA-4$^{-/-}$ mice. TGF-β is synthesized by different types

[1]*Abbreviations used in this paper:* 7-AAD, 7-amino-actinomycin D; B6, C57BL/6; CTLA-4, CTL-associated antigen 4.

of cells, including T cells (20, 25, 32), although the molecular basis and pathway(s) involved in T cell production of TGF-β are less clear.

Based on the overlapping manifestations in mice deficient in either TGF-β1 or CTLA-4 and their shared immunosuppressive actions, we sought in this study to examine whether TGF-β is involved in CTLA-4 signaling of T cells. Our results demonstrate that the cross-linking of CTLA-4 is an effective inducer of TGF-β production by murine $CD4^+$ T cells in vitro. TGF-β produced by $CD4^+$ T cells, in turn, partially suppresses the T cell proliferative response. These findings establish a previously unrecognized link between CTLA-4–mediated T cell suppression and TGF-β.

Materials and Methods

Animals. C57BL/6 (B6) mice were purchased from The National Cancer Institute, National Institutes of Health (Bethesda, MD). TGF-$\beta1^{-/-}$ mice were generated by disruption of the TGF-β1 gene in murine embryonic stem cells by homologous recombination (30). BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a specific pathogen–free rodent facility at the National Institute of Dental Research.

Antibodies and Reagents. Hamster unconjugated or PE-conjugated anti–murine antibodies to CTLA-4 (clone UC10-4F10-11), CD3 (clone 145-2C11), CD28 (clone 37.51), and purified IgG isotypic control antibody (clone G235-2356) were purchased from PharMingen (San Diego, CA). Rat anti–murine FITC-CD4 (clone CT-CD4), FITC-CD8 (clone CT-CD8a), and the respective isotypic control mAbs were purchased from Caltag Laboratories, Inc. (San Francisco, CA). Mouse anti–TGF-β1, 2, 3 mAb was from Genzyme Corp. (Cambridge, MA). Goat anti–hamster IgG (heavy and light chains) antibody was from Jackson ImmunoResearch Laboratory (West Grove, PA) and from Pierce Chemical Co. (Rockford, IL). Crystallized chicken OVA was purchased from Sigma Chemical Co. (St. Louis, MO). The following reagents were also from PharMingen: purified rat anti–mouse IL-2 (clone JES6-1A12), IL-4 (clone BVD4-1D11), and IFN-γ (clone R4-6A2) mAbs; biotinylated anti–mouse IL-2 (clone JES6-5H4), IL-4 (clone BVD6-24G2), and IFN-γ (clone XMG 1.2) mAbs; and recombinant IL-2, IL-4, and IFN-γ.

Preparation of $CD4^+$ T Cells. Spleens of B6 and asymptomatic TGF-$\beta^{-/-}$ mice were harvested, and the tissues were gently minced in HBSS supplemented with 5% fetal bovine serum (FBS; BioWhittaker, Inc., Walkersville, MD). Cells were then passed through a cell strainer (Becton Dickinson, Franklin Lakes, NJ), and red blood cells were lysed with ACK lysing buffer (BioWhittaker, Inc.). For $CD4^+$ T cell isolation, spleen cells were first passed through a nylon wool column and the nonadherent cells were further purified by using a mouse $CD4^+$ T Cell Column system (R&D Systems, Minneapolis, MN). By FACS® analysis, the purity of $CD4^+$ T cells was >95% with no detectable B cells, monocytes, or $CD8^+$ T cells. T cell–depleted spleen cells of normal BALB/c mice, irradiated at 3,000 rad, were used as APCs.

$CD4^+$ T Cell Line and Clones. A $CD4^+$ T cell line (CW-SW-1) and $CD4^+$ T cell clones 2F9 (Th0), 1A11 (Th0), 1G3 (Th2), and 1C5 (Th1) specific for chicken OVA peptide 323–339 were generated from BALB/c mice adoptively transferred with $CD4^+$ T cells of OVA TCR transgenic mice (33; a gift from Dr. D. Loh, Washington University School of Medicine, St. Louis, MO) in a tolerance model by intrathymic injection of antigen (34). All the clones express the specific clonotypic TCR (Vβ 8.2) determined by mAb KJ 1–26. Mice were injected intrathymically with OVA or PBS followed by adoptive transfer of transgenic $CD4^+$ KJ $1–26^+$ T cells and immunized 48 h later with OVA and CFA (Difco Laboratories, Inc., Detroit, MI [34]). On day 5 after immunization, the draining lymph nodes from these mice were restimulated in vitro with OVA (100 μg/ml) for 3 d and expanded with recombinant human IL-2 (10 U/ml, Boehringer Mannheim, Indianapolis, IN) for an additional 3 d. This $CD4^+$ T cell line (CW-SW-1) specifically proliferated to OVA peptide 323–339 restimulation in the presence of normal BALB/c splenic APCs. Limiting dilution cloning was carried out by stimulating the cell line CW-SW-1 (1 or 0.1 cells/well) with irradiated BALB/c splenic APCs (5×10^5/well) and OVA (100 μg/ml) in the presence of rIL-2 (10 U/ml) in 96-well flat-bottomed tissue culture plates (Costar Corp., Cambridge, MA). 7 d later, rIL-2 (10 U/ml) was added into each well and the cells were cultured for an additional 10–14 d. $CD4^+$ KJ $1–26^+$ clones were verified with the respective mAbs (34) and restimulated with OVA (100 μg/ml) for their cytokine profile. Representative Th0, Th2, and Th1 clones were chosen for this study (Table 1). The T cell clones were maintained by repetitive stimulation and resting cycles at 1–2-wk intervals by 100 μg/ml of OVA and splenic APCs of BALB/c mice.

Induction of TGF-β. Flat-bottomed 96-well plates (Costar Corp.) were coated with anti-CD3 (1 μg/ml) only or with anti-CD28 (10 μg/ml) mAbs in 100 μl PBS for 2 h at 37°C, then washed extensively and blocked for 30 min at 37°C with complete DMEM containing 10% (vol/vol) heat-inactivated FBS, 2 mM glutamine, 15 mM Hepes, 1% nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 μM 2-ME (all from BioWhittaker, Inc.). $CD4^+$ T cells (4×10^5/well) or T cell clone cells (10^5/well) were added in 200 μl of serum-free medium X-Vivo-20 (BioWhittaker, Inc.). For cross-linking assays, anti–CTLA-4 or control hamster IgG antibodies were added at 20 μg/ml or as indicated. Polyclonal goat anti–hamster antibody was then added at a final concentration of 20–40 μg/ml. For assaying antibody activity without cross-linking, anti–CTLA-4 or control hamster IgG antibodies were added at 20 μg/ml without the second goat anti–hamster antibody. For the induction of TGF-β with antigen, the $CD4^+$ T cell line CW-SW-1 was incubated with OVA (100 μg/ml) and irradiated BALB/c splenic APCs in the presence of anti–CTLA-4 or control hamster IgG antibodies (40 μg/ml) in X-Vivo-20 medium. All cultures were incubated at 37°C for 72 h, and the supernatants were collected and stored at −70°C before determination of TGF-β.

T Cell Proliferation and Cytokine Induction. The T cell activation assays were performed as described (7) with modifications where indicated. $CD4^+$ T cells were incubated with the indicated antibodies in 200 μl of complete DMEM in round-bottomed 96-well plates. Anti-CD3 was added at a final concentration of 2 μg/ml, anti-CD28 at 5 μg/ml, and anti–CTLA-4 or control hamster IgG at 20 μg/ml. For cross-linking, polyclonal goat anti–hamster antibody was added at a final concentration of 20 μg/ml. For the T cell proliferation assay, cells were then cultured at 37°C and 5% CO_2 for 72 h and pulsed with 1 μCi of [^{3}H]thymidine (NEN Research Products, Boston, MA) for the last 8–16 h. Incorporated radioactivity was counted in a beta counter (Wallac, Gaithersburg, MD). For cytokine induction, cell-free supernatants were collected at 48 h for the determination of IL-2, IFN-γ, and IL-4

by ELISA. In some experiments, anti–TGF-β1, 2, 3 (20 μg/ml) and the isotypic control antibodies were added into wells at the beginning of the culture.

Cytokine ELISAs. Quantitative ELISAs for IL-2, IFN-γ, and IL-4 were performed using paired mAbs specific for the corresponding cytokines according to the manufacturer's recommendation (PharMingen). A standard curve was generated using known amounts of purified recombinant murine IL-2, IFN-γ, and IL-4 (PharMingen). TGF-β in the supernatant was determined by the TGF-β1 Emax™ ImmunoAssay System (Promega Corp., Madison, WI) and by ELISA (25).

Flow Cytometry Analysis. $1–5 \times 10^5$ cells were resuspended in PBS without Ca^{2+} and Mg^{2+} (BioWhittaker, Inc.) containing 1% BSA (Irvine Scientific, Santa Ana, CA) and 0.1% sodium azide (Sigma Chemical Co.) (PBS-Az). For the staining of surface antigens, cells were incubated with FITC-conjugated anti–murine CD4 and PE-conjugated anti–CTLA-4 mAbs or their isotypic negative control antibodies as indicated on ice for 30 min. After washing twice with 1 ml of PBS-Az, cells were resuspended in 0.5 ml PBS-Az and analyzed on a FACScan® (Becton Dickinson). 10^4 events were routinely collected and analyzed using Lysis II software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Both the percentage of positive cells and the mean fluorescence intensity of the cells were determined.

Analysis of Cell Viability. For cell viability assays (8), T cells were cultured exactly as for the proliferation assay. After 24 and 72 h of culture, cell viability was examined by the addition of one-tenth volume of 0.4% trypan blue, and cell numbers incorporating or excluding trypan blue were determined using a hemocytometer. SDs of the duplicate wells were typically <10%.

Staining DNA of Apoptotic Cells with 7-AAD. Staining of apoptotic cells with 7-amino-actinomycin D (7-AAD) purchased from Calbiochem Corp. (La Jolla, CA) was performed by the method described by Schmid et al. (35). In brief, cells were first stained for surface antigen with FITC-conjugated anti-CD4 mAb on ice for 30 min. After washing, cells were incubated with 20 μg/ml 7-AAD in PBS-Az for 20 min at 4°C protected from light. Cells were then analyzed by FACScan® without further washing using log scale for FL-3 acquisition to assess 7-AAD staining. A minimum of 10^4 events was collected on each sample. Multiparameter data analysis was performed with Lysis II software. $CD4^+$ T cells were gated, and 7-AAD staining on FL-3 versus forward scatter channels was displayed. 7-AAD staining is divided into $7\text{-AAD}^{negative}$, 7-AAD^{dim}, and 7-AAD^{bright}, representing live, early apoptotic, and later apoptotic or dead cells, respectively.

Statistical Analysis. Statistical significance of data was analyzed by Student's *t* test.

Results and Discussion

Cross-linking of CTLA-4 with CD3 and CD28 Inhibits T Cell Proliferation and Cytokine Production. Stimulation of freshly purified $CD4^+$ T cells from naive B6 mice in the presence of anti-CD28 enhances cell growth, and as reported (2, 6–8), cross-linking of CTLA-4 together with the CD3–TCR complex and CD28 effectively inhibits T cell proliferation (Fig. 1 *A*) and IL-2 production (Fig. 1 *B*). Moreover, we also show CTLA-4–mediated reduction of IFN-γ (Th1) and IL-4 (Th2) (Fig. 1, *C* and *D*). Similar results were obtained using $CD4^+$ Th1 and Th2 clones for these studies,

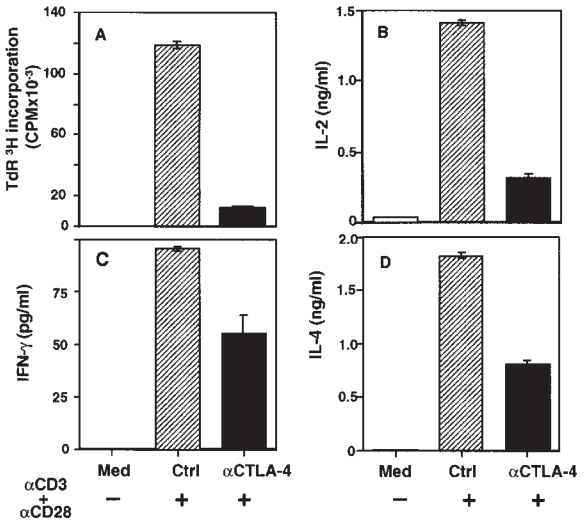

Figure 1. Cross-linking of CTLA-4 inhibits cytokine production by $CD4^+$ T cells. $CD4^+$ T cells isolated from spleens of B6 mice were cultured in complete DMEM only (*Med*) or with the indicated antibodies: anti–CTLA-4 (20 μg/ml) or control hamster IgG (*Ctrl*; 20 μg/ml) in the absence or presence of anti-CD3 (2 μg/ml) and anti-CD28 (5 μg/ml). Goat anti–hamster IgG (heavy and light chains) antibody was then added to all the wells at 20 μg/ml. T cell proliferation (*A*) was expressed as mean ± SD of triplicate wells for ^3H incorporation (*CPM*). Secretion of IL-2 (*B*), IFN-γ (*C*), and IL-4 (*D*) by $CD4^+$ T cells is shown. Supernatants were collected at 48 h, and the cytokine levels were determined by ELISA. The values are expressed as mean ± SD of replicate wells of ELISA plates.

suggesting a common pathway of suppression (Chen, W., and S.M. Wahl, unpublished results). Consistent with previous reports (2, 8), the inhibition of $CD4^+$ T cell activation by engagement of CTLA-4 could not be attributed to enhanced cell death. No significant increase in apoptosis of $CD4^+$ T cells cross-linked by anti–CTLA-4 mAb was detected either by staining of apoptotic cells with DNA dye 7-ADD for flow cytometry (Fig. 2 *A*) or by quantifying viable and nonviable cells (Fig. 2 *B*) at 24–72 h after cell culture. These data implicate alternative mechanisms of suppressed cell growth.

Cross-linking of CTLA-4 Induces TGF-β Production. To determine whether TGF-β was involved in the suppression, $CD4^+$ T cells purified from spleens of naive B6 mice were added to wells containing immobilized anti-CD3 mAb. More than 90% of these $CD4^+$ T cells are $CD44^-$, $CD69^-$, $CD45RB^{hi}$, and $CD62L^+$ and failed to proliferate to immobilized anti-CD3 mAb alone (data not shown), consistent with their naive/resting status. Although anti-CD3 mAb did not increase detectable TGF-β in the supernatant compared with that of cells cultured without anti-CD3 (Fig. 3 *A*), more than a fourfold increase in TGF-β levels above the medium control was induced by the addition of anti–murine CTLA-4 mAb followed by cross-linking with goat anti–hamster antibody. This effect was CTLA-4 specific and not due to the nonspecific binding of immunoglobulin, because the control hamster antibody had no such effect. Antibody cross-linking of CD3 and CD28, a regimen for optimal activation of naive T cells (36), failed to significantly upregulate

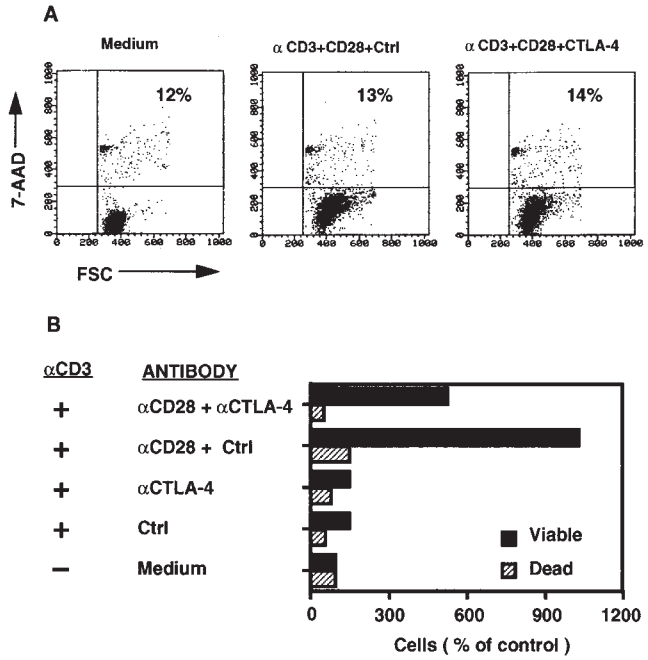

Figure 2. Cross-linking of CTLA-4 does not enhance T cell death. $CD4^+$ T cells were activated with the same antibody regimen as described for Fig. 1 *A*. (*A*) After 24 h, cells were stained with FITC–anti-CD4 antibody and 7-AAD. $CD4^+$ T cells were gated and 7-AAD staining on FL-3 versus forward scatter channels was displayed. (*B*) Cultured cells were removed and trypan blue was added (reference 8). The numbers of viable (trypan blue excluding) or dead cells (trypan blue positive) in the control medium culture were considered as 100%, and the percentage of viable and dead cells in the treated wells was calculated. The data shown here are means of duplicate wells, and the variations of duplicate wells are <10%. Data in the figure are cells after 72 h of culture. Similar results were obtained after 24 and 48 h of cell culture (not shown).

TGF-β production. However, when anti–CTLA-4 mAb was added to $CD4^+$ T cells stimulated with immobilized anti-CD3 together with anti-CD28, a marked (10-fold) increase in TGF-β occurred (Fig. 3 *A*). Cross-linking CTLA-4 without CD3–TCR complex failed to increase TGF-β production (data not shown).

Two potential mechanisms may account for the TGF-β production induced by anti–CTLA-4 antibody: stimulation of CTLA-4 signaling through antibody cross-linking or, alternatively, blockade of CTLA-4 signaling by the antibody. Since we used a goat anti–hamster antibody for cross-linking, the possibility of CTLA-4 signal transduction was favored. To further examine this hypothesis, purified $CD4^+$ T cells were stimulated with the same regimen as for Fig. 3 *A*, but without the goat anti–hamster antibody. No TGF-β was detected in the supernatants of these cell cultures (data not shown), consistent with a requirement for CTLA-4 cross-linking to signal TGF-β production. CD3, which does not directly induce TGF-β production, may nonetheless facilitate CTLA-4–induced TGF-β secretion. Although CD3 alone only minimally enhanced CTLA-4 (6; Fig. 3 *B*), CD3 plus CD28 stimulation markedly increased the expression of CTLA-4 on $CD4^+$ T cells (Fig. 3 *B*), thereby promoting CTLA-4 signaling and TGF-β induction.

Engagement of CTLA-4 in $CD4^+$ T Cell Clones Induces TGF-β. Since it was evident that engagement of CTLA-4 triggered TGF-β production in naive/resting T cells, we next analyzed whether cross-linking of CTLA-4 provided a sufficient signal to induce TGF-β production by effector/memory T cells (37) represented by murine $CD4^+$ T cell clones generated from a tolerance model by intrathymic injection of antigen (Chen, W.J., manuscript in preparation). Clone 1G3, which expresses clonotypic TCR specific to chicken OVA peptide 323–339 as determined by mAb KJ 1–26 (33, 34), produces antigen-specific IL-4 but no IFN-γ, and is characterized as a Th2 clone (Table 1). Peak surface expression of CTLA-4 on clone 1G3 was observed at 48 h after restimulation with anti-CD3 antibody or OVA in the presence of syngeneic irradiated BALB/c splenic APCs (data not shown). Similar to naive $CD4^+$ T cells, no detectable TGF-β was found in the supernatants of the clone cells, whether stimulated with immobilized anti-CD3 alone or with anti-CD28 antibodies. Surprisingly, cross-linking of CTLA-4 together with CD3 resulted in the highest level of TGF-β production (Fig. 4). In contrast to naive/resting $CD4^+$ T cells, cross-linking of CTLA-4 with CD3

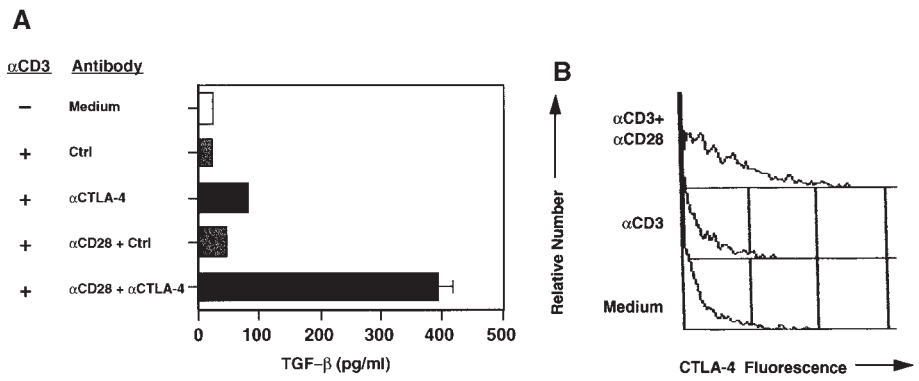

Figure 3. CTLA-4 expression and cross-linking to induce TGF-β. (*A*) Purified naive $CD4^+$ T cells were added to 96-well plates coated with anti–murine CD3 (1 μg/ml) without or with anti-CD28 (10 μg/ml). Anti–murine CTLA-4 or its isotypic control hamster IgG was added into the cultures at 20 μg/ml followed by goat anti–hamster IgG antibody (20 μg/ml). Cells were incubated in serum-free medium for 72 h. TGF-β in the supernatants was determined by ELISA. The data are expressed as mean ± SD of replicate wells (absence of SD bars indicates <10% of mean). The data shown here are representative of three experiments. (*B*) $CD4^+$ T cells were stimulated with immobilized anti-CD3 (1 μg/ml) without or with anti-CD28 (10 μg/ml) antibodies as indicated for 48–56 h. Cells were then stained with FITC–anti-murine CD4 and PE-conjugated anti-CTLA-4 mAbs. Cells were analyzed on a FACScan® with Lysis II software. Data shown are CTLA-4 fluorescence as expressed in FL-2 histograms. Fluorescence histograms of negative control PE–hamster IgG antibody, as well as CTLA-4 expression on freshly isolated $CD4^+$ T cells (data not shown), are not different from medium-treated $CD4^+$ T cells.

Table 1. *Cytokine Profile of CD4+ T Cell Clones*

Clone	Phenotype (CD4+, KJ 1-26+)	IFN-γ*	IL-4*	Th
	%	*pg/ml*	*pg/ml*	
1G3	100	<15	307	Th2
1C5	100	574	<5	Th1
2F9	100	1,843	279	Th0
1A11	100	101	186	Th0

*Cells were stimulated with OVA (100 μg/ml) in the presence of splenic APCs from BALB/c mice. Cytokine levels in 48-h supernatants were determined by ELISA.

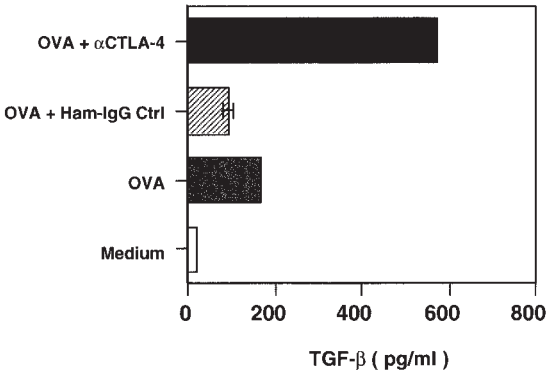

Figure 5. Engagement of CTLA-4 with antibody and stimulation of TCR by antigenic peptide upregulates TGF-β secretion. CD4+ T cell line CW-SW-1 specific for OVA peptide 323–339 was restimulated with OVA antigen (100 μg/ml) in the presence of normal BALB/c splenic APCs in X-Vivo-20. Anti–CTLA-4 (40 μg/ml) or the isotypic control hamster IgG (*Ham-IgG Ctrl*; 40 μg/ml) was added into the indicated culture wells. The supernatants were collected 72 h later and TGF-β was determined by ELISA.

plus CD28 did not further augment TGF-β levels compared with supernatants of the clone cells stimulated with anti–CTLA-4 and anti-CD3 (Fig. 4 *A*). Similar results were obtained when the OVA-specific CD4+ Th1 clone 1C5 and Th0 clone 2F9 were cultured with the same regimen of antibody stimulation (Fig. 4 *A*). These results indicate that the induction of TGF-β by CTLA-4 signaling is probably a ubiquitous feature of murine CD4+ T cells. A clear dose dependence of CTLA-4 antibody was found in the induction of TGF-β by CD4+ T cells. With increasing concentrations of anti–CTLA-4 antibody, TGF-β production was proportionally enhanced (Fig. 4 *B*).

The discrepancy in the requirement for CD28 for CTLA-4–induced TGF-β production between naive and effector (clone) CD4+ T cells is of interest. It is possible that the requirement for costimulator CD28 during restimulation of CD4+ effector T cells is greatly diminished (38) compared with naive CD4+ T cells (37). Optimal activation and, therefore, CTLA-4 expression of CD4+ naive T cells require the coengagement of CD3–TCR complex and CD28 (2, 6; Fig. 3 *B*), whereas signaling of effector cells via CD3–TCR with the respective mAb appears independently sufficient to activate CD4+ T cell clones (37) and CTLA-4 expression. Costimulation of CD28 failed to further increase CTLA-4 expression (data not shown).

Cross-linking of CTLA-4 Upregulates TGF-β Secretion in CD4+ T Cells Stimulated by Peptide Antigen. In the next series of experiments, we examined the effects of anti–CTLA-4 cross-linking on TGF-β production by T cells stimulated with antigen in the presence of syngeneic APCs. The CD4+ T cell line CW-SM-1, specific to OVA peptide 323–339, was restimulated with OVA and irradiated splenic APCs in the presence of anti–CTLA-4 or isotypic hamster IgG control antibodies. Under these conditions, CTLA-4 can be cross-linked by anti–CTLA-4 via its Fc fragment binding to Fc receptors on APCs (2, 6), which markedly enhanced antigen-specific TGF-β production (Fig. 5). It was noted that low but detectable TGF-β was also found in the supernatants of cells stimulated with anti-

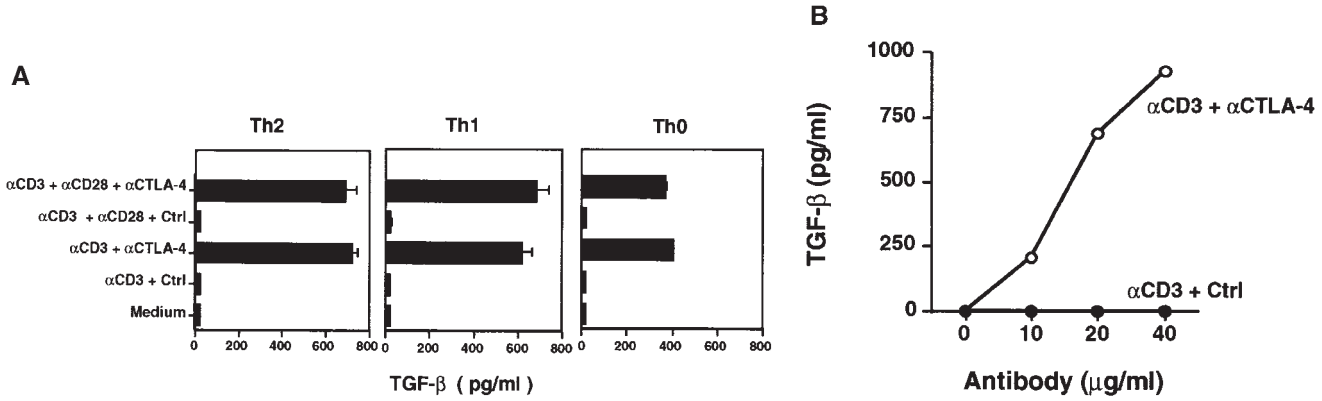

Figure 4. CTLA-4 dose-dependent induction of TGF-β. (*A*) CD4+ Th2, Th1, and Th0 cell clones specific for OVA peptide 323–339 were restimulated with the antibody regimen as described for Fig. 3 *A*. TGF-β was determined by ELISA. The data are expressed as mean ± SD of duplicate cultures. (*B*) The CD4+ T cell clone 1A11 (Th0, Table 1) was stimulated with immobilized anti-CD3 (1 μg/ml) and different concentrations of anti–CTLA-4 or isotypic hamster IgG antibodies for 72 h. Supernatant TGF-β was determined in duplicate by ELISA, and the variations of the means are <10%.

gen and APCs in the absence of anti–CTLA-4 mAb. It was unlikely that this TGF-β induced by OVA stimulation was derived from the irradiated APCs in the culture, since no TGF-β was detected in the cultures of the T cell line and APCs without the antigen (Fig. 5). Rather, the TGF-β induced by antigen and APCs can more likely be attributed to the engagement of CTLA-4 by its natural B7 family ligands on APCs. However, whether and how these B7-1 (CD80), B7-2 (CD86), and/or B7x (3, 5, 15, 36) molecules contribute to TGF-β production by engaging CTLA-4 signaling remain to be determined. Consistent with our previous data, addition of anti-CD28 mAb in this system failed to upregulate OVA-specific TGF-β production (data not shown). Importantly, these results demonstrate that cross-linking/engagement of CTLA-4 is able to induce TGF-β production by $CD4^+$ T cells stimulated with peptide antigen presented by APCs, as well as with specific antibody.

Inhibition of $CD4^+$ T Cell Activation by Engagement of CTLA-4 Is Associated with Increased TGF-β. A critical question that arises is whether the suppression of T cell activation caused by CTLA-4 engagement can, in fact, be attributed to the emergence of TGF-β. Since TGF-β is a potent inhibitor for T cell–mediated responses (19, 21, 39) and cross-linking CTLA-4 induces substantial levels of TGF-β (Fig. 3 *A*), it is reasonable to envision that TGF-β may play a part in suppression of $CD4^+$ T cell activation initiated by CTLA-4 cross-linking. Consistent with the kinetics of TGF-β production in $CD4^+$ T cells (20), we have observed that suppression of $CD4^+$ T cell activation due to cross-linking of CTLA-4 is delayed. Similar kinetics of suppression have been reported using the same CTLA-4 cross-linking antibody (2), although a different mAb induces an earlier response (24 h; reference 8). Whether this difference represents recognition of different epitopes by the antibodies is unclear, but TGF-β appears to contribute to the suppression. First, anti–TGF-β neutralizing mAb included in the $CD4^+$ T cell proliferation assay, but not the isotypic control IgG, partially restored $CD4^+$ T cell proliferation in a dose-dependent fashion. The suppression caused by CTLA-4 engagement (12,287 cpm) was partially restored (nearly threefold) by the inclusion of anti–TGF-β antibody (32,587 cpm), although it remained lower than the control proliferative response (anti-CD3 + anti-CD28 + hamster IgG: 118,229 cpm; Fig. 6). Second, we compared $CD4^+$ T cells from TGF-$\beta1^{-/-}$ mice with their wild-type TGF-$\beta1^{+/+}$ littermates for activation with anti-CD3 and anti-CD28 mAbs together with CTLA-4 cross-linking. As shown (Fig. 1 *A*), CTLA-4 engagement profoundly inhibited T cell proliferation (90–96% suppression) in the wild-type T cells, in contrast to T cells from TGF-$\beta1^{-/-}$ mice (Fig. 7). Although TGF-$\beta1^{-/-}$ T cells are less proliferative in vitro than wild-type T cells (31) using optimal conditions of anti-CD3 and anti-CD28 antibodies for the wild-type and making comparisons difficult, we adjusted the anti-CD3 and anti-CD28 concentrations to increase proliferation of the TGF-$\beta1^{-/-}$ $CD4^+$ T cells (Fig. 7). Similar to wild-type $CD4^+$ T cells, anti-CD3 and anti-CD28 mAbs induced significant expression of CTLA-4 on the TGF-$\beta1^{-/-}$ T cells after 48 h of culture (>50% of $CD4^+$ T cells become positive; Fig. 7 *A*). However, CTLA-4 cross-linking only suppressed the TGF-$\beta1^{-/-}$ T cell proliferation by 30–40% (Fig. 7 *B*), supporting the hypothesis that TGF-β1 contributes to CTLA-4–mediated suppression of T cell activation. Since engagement of CTLA-4 in TGF-$\beta1^{-/-}$ $CD4^+$ T cells still results in some degree of suppression of T cell proliferation, other inhibitory molecules may also be associated with CTLA-4 signaling. Collectively, these data indicate that stimulation of CTLA-4 inhibits $CD4^+$ T cell activation, at least in part, by inducing TGF-β production, which then downregulates their immune responsiveness.

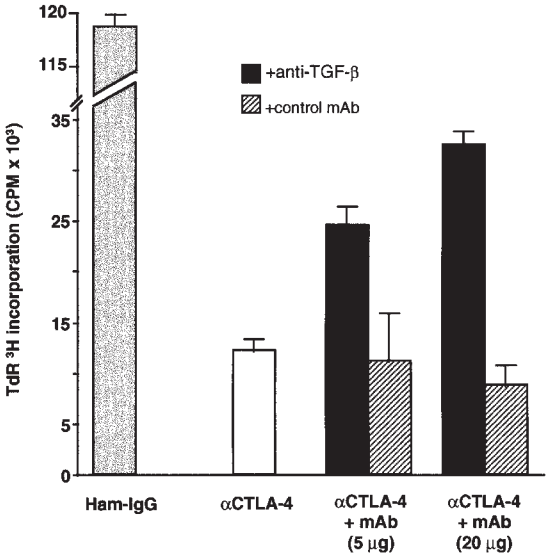

Figure 6. TGF-β is associated with inhibition of $CD4^+$ T cell activation by CTLA-4 cross-linking. $CD4^+$ T cells isolated from B6 spleens were cultured with anti-CD3 (2 μg/ml), anti-CD28 (5 μg/ml), and anti–CTLA-4 (20 μg/ml) followed by goat anti–hamster IgG (*Ham-IgG*; 20 μg/ml). Anti–TGF-β mAb or isotypic control antibody (mouse IgG1) was included from the beginning of the culture at the indicated concentrations. Cells were cultured for 72 h. Data are expressed as mean cpm ± SD of triplicate wells for [^{3}H]thymidine incorporation. Data not shown in the figure are cpm of T cells incubated with medium alone (176 ± 26). The experiments were repeated three times with similar results.

Our results that cross-linking of anti–CTLA-4 mAb induces TGF-β production, which then reduces T cell activation, offer an explanation for the mechanisms whereby the CTLA-4 molecule functions as a negative regulator of T cell responses in vitro (2, 6–8). This finding also has implications in our understanding of the function of CTLA-4 in T cell immune responses in vivo.

Despite the apparent unrelatedness of the two molecules, emerging evidence has begun to link TGF-β and CTLA-4. Genes for both CTLA-4 (2, 3) and TGF-β (for reviews, see references 39 and 40) are highly conserved among mouse, rat, and human species. Engagement of CTLA-4 results in profound inhibition of T cell activation triggered by TCR stimulation, including suppression of T cell cycle

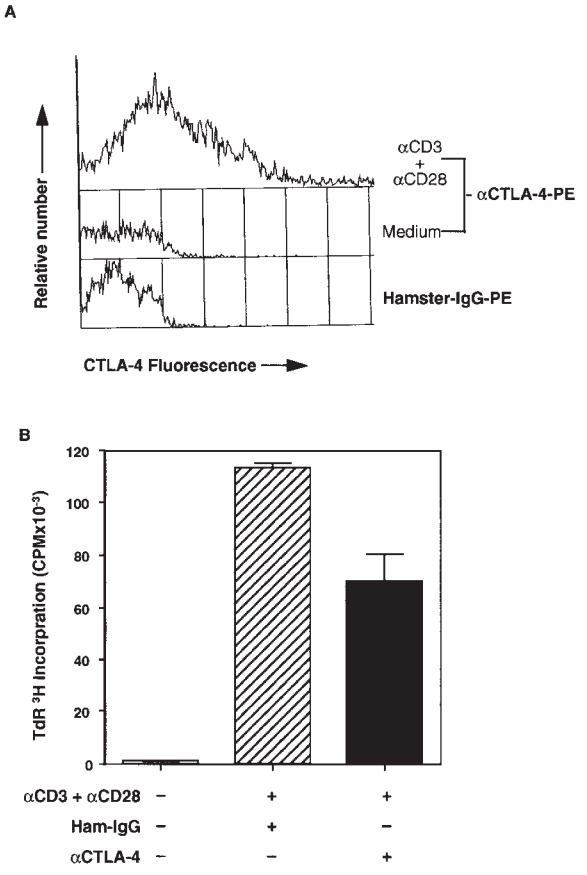

Figure 7. Defect of CTLA-4–induced suppression of $CD4^+$ T cell proliferation in TGF-$\beta1^{-/-}$ mice. Freshly purified $CD4^+$ T cells from asymptomatic TGF-$\beta1^{-/-}$ mice were stimulated with the modified antibody regimen as indicated. Anti-CD3: 0.5 μg/ml; anti-CD28: 0.2 μg/ml; anti–CTLA-4 and hamster IgG (*Ham-IgG*): 20 μg/ml. Goat anti–hamster IgG (20 μg/ml) was added to all wells. (*A*) Surface expression of CTLA-4 on treated and control TGF-$\beta1^{-/-}$ $CD4^+$ T cells by flow cytometry. Cells were harvested after 56 h of culture and stained with FITC–anti-CD4 and PE–anti-CTLA-4. $CD4^+$ T cells were gated and CTLA-4 staining of treated (*top*) and control (*middle*) cells on FL-2 channels is displayed. *Bottom*, negative control antibody staining for PE-conjugated anti-CTLA-4. (*B*) T cell proliferation of treated and control TGF-$\beta1^{-/-}$ T cells. Data are expressed as mean ± SD (*CPM*) of triplicate wells.

(2, 8), decreased IL-2 production, and reduction of both Th1 and Th2 cytokines (Fig. 1). TGF-β has a similar profile of activities (19, 20, 41). Neither engagement of CTLA-4 (2, 8, 16) nor inclusion of TGF-β (37) induces apoptosis in murine $CD4^+$ T cell cultures. Recombinant IL-2 is able to reverse the inhibition induced either by CTLA-4 cross-linking (2) or by exogenous TGF-β (20; Chen, W., and S.M. Wahl, manuscript in preparation). Most importantly, disruption of TGF-β1 (29, 30) or CTLA-4 (13, 14) genes in mice leads to massive inflammation, multiorgan lymphocyte infiltration, and spontaneous T cell activation. Both genotypes are associated with death at the age of 2–4 wk. Now our data further strengthen this link by demonstrating that CTLA-4 acts as a negative regulator by inducing TGF-β and possibly other inhibitory molecules in T cells.

In vivo, the pathways whereby anti–CTLA-4 antibody functions remain to be determined, although either blockade or stimulation of CTLA-4 by the injected antibodies may occur. Blockade of CTLA-4 signaling by the antibody may lead to a loss of TGF-β production by T cells. Since TGF-β contributes to immune tolerance, suppression of autoimmune diseases in animal models (23–25, 42–44), and inhibition of antitumor immunity of T cells infiltrating into tumors (45–48), loss of TGF-β production and removal of this potent inhibitor of T cells could release a "brake" on T cell activation. Consequently, augmentation of the T cell antitumor immune response (11) and exacerbation of autoimmune diseases such as experimental autoimmune encephalomyelitis due to increased IL-2 and IFN-γ production may occur (3, 12). On the other hand, if anti–CTLA-4 antibody given in vivo cross-links CTLA-4 on T cells via its Fc fragment on FcR^+ cells, T cells might commence production of TGF-β to effect immune suppression, tolerance (49), and inhibition of tumor growth (50–52). Thus, based on our in vitro data, it is now possible to reevaluate the in vivo impact of CTLA-4 antibodies on TGF-β production and their cooperative roles in mediating antitumor responses, immunoregulation, and tolerance.

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