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Expression and Function of CTLA-4 in Th1 and Th2 Cells

Maria-Luisa Alegre,*† Helena Shiels,* Craig B. Thompson,*† and Thomas F. Gajewski†‡

CTLA-4 is expressed on T cells after activation and shares homology with the CD28 costimulatory receptor. In contrast to CD28, CTLA-4 is thought to be a negative regulator of T cell activation. Cross-linking of CTLA-4 during activation of peripheral T cells reduces IL-2 production and arrests T cells in G1. Much less is known about the function of CTLA-4 in differentiated T cells. We have investigated the expression and function of CTLA-4 in established Th1 and Th2 clones and in bulk populations of Th1 and Th2 cells freshly derived in vitro from TCR transgenic splenocytes. We found that CTLA-4 was induced under similar conditions and with similar kinetics following activation of both Th1 and Th2 clones. However, CTLA-4 expression was much higher in Th2 than Th1 clones and lines. This was confirmed by flow cytometry, confocal microscopy, and Northern blot analysis. The ratio of surface to intracellular expression of CTLA-4 and its rate of endocytosis were similar in Th1 and Th2 clones. Inhibition of binding of CTLA-4 to its ligands using soluble anti-CTLA-4 mAb during stimulation with Ag increased the production not only of IL-2 by Th1 clones, but also that of IL-3 and IFN-γ by Th1 clones and of IL-3, IL-4, IL-5, and IL-10 by Th2 clones. In contrast, when anti-CTLA-4 was coimmobilized with anti-CD3 and anti-CD28 mAbs, a decrease in the production of multiple cytokines was observed. We conclude that CTLA-4 can function to suppress the production of cytokines produced by both Th1 and Th2 cells.


T cell activation is initiated by the concurrent engagement of the TCR and of a costimulatory molecule such as CD28. CTLA-4 (CD152) shares some homology with CD28 and binds to the same ligands, namely CD80 (B7-1) and CD86 (B7-2) (1). However, unlike CD28, CTLA-4 is not expressed in naive T cells, and, when it becomes up-regulated following T cell activation, it is thought to down-modulate T cell responses rather than augment them (2). The function of CTLA-4 has been studied primarily using lymphocytes from unprimed animals, which comprise a mixture of naive and previously activated T cells. Cross-linking of CTLA-4 on these normal peripheral T cells has been shown to reduce the production of IL-2 and prevent the up-regulation of IL-2R, resulting in G1 cell cycle arrest, both in murine and human lymphocytes (3–5). In addition, Perez et al. have recently suggested that, whereas blocking of CD28 prevents T cell priming, inhibition of CTLA-4 prevents tolerance induction in mice in vivo (6). Furthermore, administration of blocking Abs to CTLA-4 in vivo also can result in enhanced immune responses leading to rejection of otherwise progressively growing tumors (7), as well as to protection against nematode infection (8).

Following initial activation of naive T lymphocytes, CD4+ cells undergo a differentiation process that eventually can result in the generation of Th1 or Th2 cells. Th1 cells secrete IL-2 and IFN-γ upon activation whereas Th2 lymphocytes produce IL-4, IL-5, and IL-10 and are less effective than Th1 cells at cytolysis. Ligation of the TCR on Th1 cells in the absence of CD28 stimulation results in the reduced capacity of these cells to produce IL-2 and proliferate upon subsequent activation, a state termed clonal anergy (9). Th2 lymphocytes, in contrast, do not appear to alter their cytokine production phenotype under such anergizing conditions. A number of pathologic situations in vivo seem to result from a Th1 or Th2 predominance. For instance, failure to cure Leishmania infection is observed in mice that generate a predominantly Th2-type response to the pathogen, whereas strains of mice that preferentially give rise to Th1 cells display a healer phenotype (10). In contrast, Th1-type responses seem to be the driving force behind development of autoimmune diabetes in nonobese diabetic (NOD) mice (11). Thus, a better understanding of the regulation of Th1 and Th2 function might allow intervention in a number of immunopathologic situations in vivo.

The initial characterization of expression and function of CTLA-4 has been performed using normal peripheral T cells. The function of CTLA-4 in differentiated T cells is not known. Because Th1 but not Th2 cells are susceptible to anergy induction and inasmuch as CTLA-4 may be important for the induction of a tolerant state, the expression and function of CTLA-4 in Th1 and Th2 cells was investigated. Initial attempts at detecting expression of CTLA-4 by flow cytometry in T cell clones had been unsuccessful, apparently because of the low level of CTLA-4 found on the cell surface (12). This appeared to be at odds with studies showing expression of CTLA-4 mRNA by Northern blot analysis (13). However, more recent studies have revealed that the majority of CTLA-4 is localized in intracellular stores rather than on the cell surface, because CTLA-4 is rapidly endocytosed away from the cell surface (14, 15). Thus, we have readdressed CTLA-4 expression using intracellular staining techniques on established T cell clones and freshly derived bulk populations of T helper cells. In addition, we have compared the functional properties of CTLA-4 in Th1 and Th2 clones.

Materials and Methods

Mice

DO11.10/BALB/c mice, the transgenic TCR of which is specific for a peptide derived from chicken OVA and presented by I-Ad, were generously provided by Dr. Kenneth M. Murphy (Washington University, St. Louis, MO). CD28-deficient mice were generated as previously described (16) and back-crossed to BALB/c mice for 6 generations. DBA/2 and BALB/c mice were obtained from Jackson ImmunoResearch Laboratories (West Chester, PA).
The hybridomas secreting the hamster anti-murine CTLA-4 mAb 4F10 (17) and anti-murine CD3 mAb 145-2C11 (18) were generously provided by Jeffrey A. Bluestone (University of Chicago). The Abs were purified from culture supernatant by binding to protein A (Sigma, St. Louis, MO). The hamster anti-mouse CD28 mAb PV-1 was purified and kindly provided by Carl June (Naval Medical Research Institute, Bethesda, MD). A mixture of monoclonal hamster IgGs (Cappel, Durham, NC) was used as a control Ab for 4F10, 145-2C11, and PV-1. Recombinant human IL-2 and recombinant murine IL-4 were purchased from Boehringer Mannheim (Indianapolis, IN). Anti-IL-4 and anti-IL-12 mAbs were obtained from PharMingen (San Diego, CA). OVA protein was obtained from Sigma. OVA peptide was synthesized by Multiple Peptide Systems (San Diego, CA). Abs for flow cytometry (anti-CD4 FITC, phycoerythrin (PE)2-coupled anti-CTLA-4, and control hamster IgG-PE), as well as all the Ab pairs for cytokine ELISA, were all purchased from PharMingen.

Reagents

The Th1 clone pG110 and the Th2 clone pL104 have been described and characterized previously (19). T helper clones were cultured in high glucose DMEM (Life Technologies, Grand Island, NY) supplemented with 5% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), MOPS, β-mercaptoethanol (50 μM), and additional amino acids. The clones (10⁶/well) were passaged weekly in the presence of OVA, recombinant human IL-2 (10 U/ml), and irradiated (2000 rad) DBA/2 splenocytes (6 × 10⁶/well) in 24-well plates (Linbro, Aurora, OH). For experiments, cells were collected and adjusted to a density of dead cells by density gradient centrifugation through Ficoll-Hypaque (Cedarlane Laboratories, Hornby, Ontario, Canada). For induction of CTLA-4 expression and detection of cytokines, Th clones (5 × 10⁵ cells) were stimulated with OVA (500 μg/ml) and irradiated DBA/2 splenocytes for the indicated times with or without hamster IgG (50 μg/ml) or anti-CTLA-4 mAb (50 μg/ml). For detection of cytokine production after CTLA-4 cross-linking, Th clones or bulk populations of Th cells (generated as described below) were incubated in plates coated with goat anti-hamster IgG (10 μg/ml) followed by anti-CD3 mAb (1 μg/ml), anti-CD28 mAb (1 μg/ml), and hamster IgG (50 μg/ml) or anti-CTLA-4 mAb (50 μg/ml). Supernatants were collected at 24 and 48 h and stored at −20°C until tested.

Fresh bulk populations of Th1 and Th2 lines were obtained by incubating D011.10 splenocytes (2 × 10⁶ cells/well in 2 ml of complete medium in 24-well plates) in the presence of OVA peptide (0.5 μM) and either IL-12 (2 ng/ml) + anti-IL-4 (10 ng/ml) or IL-4 (2 × 10³ U/ml) + anti-IL-12 (10 μg/ml) to obtain Th1 and Th2 lines, respectively. Cells were harvested after 6 days, and dead cells were removed by centrifugation through Lympholyte M (Cedarlane Laboratories). For induction of CTLA-4 expression, 1-wk-old Th1 and Th2 lines (250 × 10³/well in 2 ml of complete medium in 24-well plates) were stimulated with OVA peptide (2 μM) in the presence of irradiated (2000 rad) CD28-deficient BALB/c syngeneic splenocytes for 72 h.

Flow cytometry and confocal microscopy

Two-color flow cytometry on murine clones and cell lines to detect surface expression of CTLA-4 was performed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA), interfaced to a Macintosh computer. Data analysis was performed using Cell Quest software (Becton Dickinson). Logarithmically amplified fluorescence data were collected on 10,000 CD4+ cells. All flow cytometry staining procedures, except for the endocytosis experiments, were performed at 4°C in flow cytometry buffer (1× PBS, 0.01% NaN₃, 1% BSA; Sigma). For intracellular detection of CTLA-4, Th clones were first surface stained using anti-CD4 FITC mAbs at 4°C, fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA), washed in 0.03% saponin buffer (Sigma), and stained with control hamster IgG-PE or anti-CTLA-4-PE at 4°C in the presence of 0.3% saponin and normal goat serum (Vector Laboratories, Burlingame, CA). Cells were then extensively washed in 0.03% saponin and flow cytometry buffer before analysis by flow cytometry or by confocal microscopy. For the endocytosis experiments, cells were incubated at 37°C in complete medium in the presence of the relevant staining Abs, for 30 min. Internalization was stopped with cold flow cytometry buffer, and the cells were centrifuged, surface stained at 4°C with 2 Abbreviations used in this paper: PE, phycoerythrin; Erk, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

### FIGURE 1

CTLA-4 is expressed at higher levels in Th2 than in Th1 clones. A, The Th1 clone pG110 and the Th2 clone pL104 were incubated in the presence or absence of recombinant human IL-2 (10 U/ml), OVA Ag (800 μg/ml), and irradiated (2000 rad) syngeneic splenic APCs. Cells were harvested at 48 h, depleted of dead cells by centrifugation through a Ficoll-Hypaque gradient, surface stained with anti-CD4 FITC, stained intracellularly with control hamster IgG-PE or anti-CTLA-4-PE, and analyzed by flow cytometry as described in Materials and Methods. Ten thousand live CD4+ cells/sample were collected. Results represent the mean fluorescence of CD4+ cells obtained with the anti-CD4-PE mAb minus that obtained with the control hamster IgG and are representative of two independent experiments. B, The Th1 clone pG110 and the Th2 clone pL104 were incubated for 48 h in the presence of OVA Ag and irradiated syngeneic splenic APCs. Cells were processed as described in A and surface stained with anti-CD4 FITC and either hamster IgG-PE or anti-CTLA-4-PE on intact (top panels) or permeabilized (bottom panels) cells. B, Shows a histogram of the mean fluorescence of CD4+ cells obtained with the control hamster IgG-PE (thin lines) and with anti-CTLA-4-PE (bold lines). The number in the upper corner represents the mean fluorescence of CD4+ cells obtained with the anti-CTLA-4-PE mAb. This result is representative of four independent experiments.
FIGURE 2. CTLA-4 is expressed with the same kinetics in Th1 and Th2 clones. The Th1 clone pGL10 and the Th2 clone pL104 were incubated with OVA and irradiated syngeneic splenic APCs as described in Figure 1. Cells were harvested at different time points after activation and processed as above. Cells were surface stained with anti-CD4 FITC and stained with hamster IgG-PE or anti-CTLA-4-PE on intact (left panel) or permeabilized (right panel) cells. Delta Mean Fluorescence stands for the mean fluorescence of CD4$^+$ cells stained with anti-CTLA-4-PE minus that observed with control hamster IgG-PE. This result is representative of two independent experiments.

FITC-anti-Thy-1 mAb, and finally fixed in 1% paraformaldehyde. Cells stained for flow cytometry analysis were visualized for immunofluorescence by confocal microscopy using a Zeiss LSM 410 system equipped with a 100x objective. Two optical sections (0.75–1 μm) were obtained consecutively (red 568 nm excitation/590 nm emission filter, green 488 nm excitation/515 nm emission filter) and overlaid to provide two-color images. Data were analyzed using LSM software (Zeiss, Oberkochen, Germany) and presented using Photoshop software (Adobe, Mountain View, CA).

Northern blot analysis

Forty-eight hours after stimulation with irradiated splenocytes and OVA, Th clones or bulk populations of Th lines were harvested, depleted of dead cells by centrifugation through Lympholyte M, and 20 × 10^5 cells were used to extract RNA. RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) and was quantitated by optical density determination. Equalized RNA samples were separated on a 1% agarose/formaldehyde gel and transferred to nitrocellulose as previously described (12).

A 300-kb CTLA-4 cDNA probe containing the extracellular and transmembrane domains of murine CTLA-4 was labeled by nick translation and used for detection, and a 5S ribosomal RNA oligomer composed of twenty bases was used after 5′ end labeling to check for equal loading of the lanes.

Proliferation assays

Proliferation assays were performed using 5 × 10^4 T cell clones/well in 96-well plates (Costar, Cambridge, MA), in the presence of OVA Ag (800 μg/ml), irradiated DBA/2 splenocytes (1 × 10^5/well), and control hamster Ab (50 μg/ml) or anti-CTLA-4 mAb (50 μg/ml) and 20 × 10^5 T cells were used for detection, and a 5S ribosomal RNA oligomer composed of twenty bases was used after 5′ end labeling to check for equal loading of the lanes.

Cytokine ELISAs

ELISAs were performed according to the instructions of the manufacturer using purified mAbs as capture Abs and biotinylated mAbs as developing Abs, followed by incubation with streptavidin-alkaline phosphatase and substrate. Plates were read in a 96-well spectrophotometer (SpectraMax 250, Molecular Devices, Sunnyvale, CA) and data were analyzed using SoftMax software (Molecular Devices) by comparison against a standard curve generated using recombinant cytokines at known concentrations.

Results

CTLA-4 is expressed at higher levels in Th2 than Th1 clones

Ligation of CTLA-4 has been reported to inhibit the production of IL-2 by normal peripheral T cells (3–5). However, the potential ability of CTLA-4 to control the production of other cytokines and to regulate differentiated T cell subsets has not yet been examined. To begin to address whether CTLA-4 could affect T cell functions other than IL-2 production, we analyzed the expression of CTLA-4 in terminally differentiated Th1 and Th2 clones. T cell clones offer the additional advantage of being activatable with nominal Ag in addition to anti-CD3 mAb. With normal T cells, optimal CTLA-4 expression is induced 48 to 72 h following TCR stimulation, is augmented by exogenous IL-2, and the majority of the CTLA-4 expressed is present intracellularly (15, 17). The stimuli required for CTLA-4 induction in differentiated cells were not yet known. Therefore, we stimulated Th1 and Th2 clones under a variety of conditions and analyzed CTLA-4 protein expression after intracellular flow cytometry at 48 h. As shown in Figure 1, CTLA-4 was not expressed in unstimulated T cell clones but was induced following activation with irradiated syngeneic splenocytes and Ag (Fig. 1A). This expression was further increased in the presence of IL-2. However, it appeared that the Th2 clone expressed about 10-fold more intracellular CTLA-4 than the Th1 clone. We next compared surface and intracellular expression of CTLA-4 following activation of each cell type. No surface expression could be detected on the Th1 clone pGL10, whereas surface CTLA-4 was easily detected on the Th2 clone pL104 (Fig. 1B). As observed in the previous experiment, higher levels of intracellular CTLA-4 were detected in the Th2 than in the Th1 clone. Similar results were obtained with another set of Th1 (pGL2, intracellular CTLA-4 mean fluorescence intensity of 52) and Th2 clones (pL3, intracellular CTLA-4 mean fluorescence intensity of 148) (data not shown). In contrast to the differences observed in CTLA-4 expression, CD28 was found to be expressed at similar levels in the two Th1 and 2 Th2 clones studied (surface and intracellular mean fluorescence intensity between 30 and 50, data not shown).

To determine whether lower expression of CTLA-4 in Th1 than Th2 clones at 48 h could be a reflection of different kinetics in the two cell lines, we performed a time course of surface and intracellular expression of CTLA-4 in pGL10 and pL104. As shown in Figure 2, CTLA-4 was expressed with similar kinetics in the two clones, both at the surface and intracellular levels. Peak levels were reached in both cell types 24 to 48 h after stimulation, and CTLA-4 expression declined to barely detectable levels 7 days after activation. This decline in CTLA-4 expression was due presumably to quiescence of the Th clones and not to cell death since viability at day 7 was excellent (data not shown). This result suggests that CTLA-4 expression is not sustained in effector cells and that levels slowly decrease to baseline as the cells quiesce.

Western blotting confirmed the higher expression of CTLA-4 on Th2 clones, as compared with Th1 clones. As previously described (20, 21), CTLA-4 was seen as a broad band of 27 to 40 kDa due to its heavily glycosylated state. Deglycosylation of cell lysates using PNGlycanase F before CTLA-4 immunoprecipitation resulted in a band of approximately 20 kDa (data not shown).

CTLA-4 is expressed at higher levels in freshly derived bulk populations of Th2 compared with Th1 cells

It was conceivable that the difference in CTLA-4 expression was due to clonal variation among the individual clones examined rather than to the Th2 vs Th1 phenotype. To determine whether this observation was generally true, bulk Th1 and Th2 populations were generated from DO11.10 splenocytes stimulated in vitro with...
OVA peptide under Th1-inducing or Th2-inducing conditions, as described in Materials and Methods. After one week of differentiation, the resulting cells were restimulated in vitro for 48 h, and CTLA-4 expression was assessed. Figure 3 shows the profile of cytokines obtained after this restimulation. The bulk population of Th1 cells expressed high levels of IFN-γ and low levels of IL-4, whereas the bulk population of Th2 cells had the opposite phenotype. The expression of CTLA-4 by flow cytometry in the bulk populations paralleled that found in the T cell clones. Indeed, higher levels of both surface and intracellular CTLA-4 were observed in Th2 compared with the Th1 populations (Fig. 3B), suggesting that this difference was not an artifact of analyzing single clones.

Differential expression of CTLA-4 in Th clones and bulk populations of T cells is regulated at the mRNA level
To determine whether the difference in levels of CTLA-4 protein between Th1 and Th2 clones and bulk populations was transcriptionally or posttranscriptionally regulated, total RNA was extracted from pGL10 and pL104, as well as from 1-wk-old bulk Th1 and Th2 populations, 48 h after restimulation with Ag and irradiated splenocytes. Similarly to CTLA-4 protein, CTLA-4 mRNA was expressed at much higher levels in Th2 than Th1 clones and bulk populations (Fig. 4). Equal loading was checked through 5S RNA visualization. This result suggests that differential expression of CTLA-4 is, at least in part, regulated by mRNA abundance.

Th1 and Th2 clones have a similar cellular distribution of CTLA-4
We and others have shown that the majority of CTLA-4 is localized to intracellular compartments, with usually less than 20% of total CTLA-4 expressed on the cell surface (15, 21). In addition, surface CTLA-4 is rapidly cleared into intracellular endocytic vesicles, in a clathrin-dependent manner (22–24). Intracellular staining of CTLA-4 in pGL10 and pL104 cells was performed, and the stained cells were examined by confocal microscopy. As shown in Figure 5, A and B, although much more CTLA-4 was observed in the Th2 clone pL104 than in the Th1 clone pGL10, the intracellular distribution of CTLA-4 was similar in the two clones. The appearance of intracellular CTLA-4 staining in both cell types had a vesicular pattern, similar to what had been found previously using activated primary T cells (14, 15).

Nonpermeabilized pGL10 and pL104 cells were then incubated at 37°C with anti-CTLA-4-PE as previously described (15) to determine whether CTLA-4 can undergo endocytosis in Th1 and Th2
clones. Figure 5, C and D, shows that although the amount of surface CTLA-4 endocytosed in 30 min was much lower in the Th1 clone pGL10 than in the Th2 clone pL104, a substantial amount of CTLA-4 did accumulate in endocytic vesicles in both clones. This was specific to CTLA-4 since no intracellular accumulation occurred when the cells were incubated under the same conditions with hamster IgG-PE or with anti-Thy-1.2-FITC (data not shown). These results indicate that, although the levels of expression are quite different between Th1 and Th2 clones, the distribution and trafficking of CTLA-4 are similar in both types of cells.

CTLA-4 is functional in Th1 and Th2 clones and lines and regulates the production of multiple cytokines

Because the levels of expression of CTLA-4 were so low in Th1 clones, we sought to determine whether CTLA-4 was functional in both cell types. In addition, inasmuch as all previous studies of CTLA-4 function had used anti-CD3 mAb + anti-CD28 mAb as a stimulus (3–5, 17, 25), the use of Ag-specific T cell clones allowed us to examine CTLA-4 function during response to nominal Ag. The Th1 clone pGL10 and the Th2 clone pL104 were stimulated with OVA and irradiated syngeneic APCs in the presence of hamster IgG and irradiated syngeneic splenocytes in the presence of hamster IgG or of anti-CTLA-4. Supernatants were collected at 24 and 48 h, and cytokine concentrations were assayed by ELISA. When in soluble form, 4F10 anti-CTLA-4 mAb has been characterized to be a blocking Ab that disrupts binding of CTLA-4 to its ligands (17). The addition of soluble anti-CTLA-4 mAb to Th1 and Th2 cultures resulted in the reduced production of all the cytokines that were tested, but clonal specificity was maintained. Th1 clones secreted increased amounts of IL-2, IFN-γ, and IL-3 (Fig. 6), and TNF-α (data not shown), whereas Th2 clones produced a higher concentration of IL-3, IL-4, IL-5, and IL-10 (Fig. 6). However, contrary to published results using normal murine T cells (17), proliferation of Th1 and Th2 clones was only marginally increased by the addition of anti-CTLA-4 mAb (Fig. 7), and the kinetics of the rise and fall of thymidine incorporation were preserved. Thus, the increased expression of CTLA-4 seen after activation does not appear to be the main limitation to sustained proliferation in vitro.

In contrast to anti-CTLA-4 used in soluble form, cross-linked anti-CTLA-4 mAb has been shown to behave as an agonistic rather than a blocking mAb (3). To determine whether cross-linking of CTLA-4 would inhibit cytokine release, pGL10 and pL104 were stimulated with immobilized anti-CD3 + anti-CD28 mAbs in the presence of immobilized either control hamster IgG or anti-CTLA-4 mAb. Indeed, cross-linking of CTLA-4 along with TCR/CD28 stimulation resulted in the reduced production of the same cytokines, again respecting clonal specificity (Fig. 8). The production of IL-2 and IFN-γ from Th1 clones and that of IL-3, IL-4, IL-5, and IL-10 from Th2 clones was reduced, as was that of TNF-α from both Th1 and Th2 clones (data not shown). Similar results were obtained when bulk populations of DO11.10 Th1 and Th2 lines were incubated with immobilized anti-CD3, anti-CD28, and anti-CTLA-4 mAbs (Fig. 9). These data suggest that CTLA-4 is functional in both Th1 and Th2 cells and regulates the production of a broad array of cytokines.

Discussion

In contrast to CD28, CTLA-4 up-regulation following T cell activation is thought to lead to down-modulation of T cell responses (3–5, 17, 25). Indeed, mice made deficient in CTLA-4 by homologous recombination develop a lymphoproliferative disorder and an autoimmune disease leading to death of the animals by 3 to 4 wk of age (26, 27). CD28 and CTLA-4 may also play a role in Th1/Th2 differentiation. Ligation of CD28 during TCR stimulation in vitro has been reported to promote the differentiation of Th2 cells (28). In addition, T cells from CTLA-4-deficient mice have

FIGURE 5. The pattern of distribution of CTLA-4 is similar in Th1 and Th2 clones. The Th1 clone pGL10 and the Th2 clone pL104 were stimulated for 48 h with OVA and irradiated syngeneic APCs. Live cells were isolated. A and B, Cells were surface stained with Thy1.1-FITC, then fixed, permeabilized, and stained with anti-CTLA-4-PE. C and D, Intact cells were incubated at 37°C in the presence of anti-CTLA-4-PE for 30 min before fixing. Cells were examined by confocal microscopy for FL1 (green) and FL2 (red) excitation (A and B), and for FL2 (red) and transmitted light to determine the contour of the cells (C and D). This result is representative of two independent experiments.
been reported to have increased IL-4 secretion upon TCR stimulation (26). Inasmuch as predominance of a Th1- or Th2-type response has been correlated with a number of pathologic states, we found it of interest to determine whether Th1 and Th2 cells would exhibit differential CTLA-4 expression and/or function. Unexpectedly, we found that expression of CTLA-4 was much higher in Th2 than Th1 Ag-stimulated clones and bulk populations of cells, both at the mRNA and protein levels. This was probably not because Th2-derived cytokines could increase CTLA-4 expression more than would Th1-derived factors, since we had found previously that, when given concomitantly to TCR stimulation, IL-4, IL-6, or IL-10 did not markedly up-regulate CTLA-4, whereas IL-2 did (15).

Other surface molecules have been shown to behave differently in Th1 and Th2 clones. For example, Th1 clones are susceptible to Fas-mediated and activation-induced cell death whereas Th2 clones are not (29, 30). In addition, expression of CD30 has been found generally to be higher in Th2 than Th1 clones (31). Finally, established Th2 clones do not appear to require CD28 signals for their maintenance whereas Th1 clones do (32). Therefore, one
hypothesis for the higher expression of CTLA-4 in Th2 than Th1 cells could be that Th1 cells have additional mechanisms to arrest their proliferation, such as sensitivity to Fas, whereas Th2 cells may rely more on CTLA-4 function to prevent overexpansion. Higher expression of CTLA-4 in Th2 cells might have resulted in cells much more sensitive to CTLA-4-dependent down-regulation of immune responses than Th1 cells. However, despite low levels of expression in Th1 cells, CTLA-4 was functional in a similar manner in both Th1 and Th2 cells. High concentrations of anti-CTLA-4 mAb were used in these experiments in an attempt to block all CTLA-4 molecules in the Th2 clones. It is unlikely that higher concentrations of anti-CTLA-4 mAb would have resulted in a stronger inhibition of Th2 cell function. Indeed, titration by flow cytometry revealed that the Ab was saturating when used at 0.25 μg/ml, whereas a 200-fold higher concentration was utilized for the functional studies.

It has been suggested that stronger signals are necessary for Th2 than for Th1 differentiation (32, 33). Indeed, higher initial concentrations of Ag may favor Th2 development. Therefore, one could imagine also that more CTLA-4 may be needed to counteract these strong signals required for Th2 differentiation. Alternatively, higher expression of CTLA-4 might be a consequence of a low responsiveness by Th2 cells to CTLA-4 function. Little is known about the mechanism by which CTLA-4 exerts its inhibitory function. It has been speculated that CTLA-4 may scavenge B7 ligands, rendering them unable to bind CD28 and thus reducing T cell responses. If this were the case, immune responses that would result in Th2 differentiation would be more effective at blocking further T cell stimulation by reducing B7 availability. However, this may not be the only mechanism by which CTLA-4 alters immune responses. More recently, there has been evidence that the cytoplasmic tail of CTLA-4 can bind the lipid kinase phosphatidylinositol 3-kinase (PI3K) (20) and the tyrosine phosphatase Src homology 2-containing tyrosine phosphatase (SHP)-2 (34). We have shown that cross-linking of CTLA-4 resulted in down-regulation of all the cytokines that were tested, including IL-2, IL-3, IL-4, IL-5, IL-10, TNF-α, and IFN-γ. This suggests that a central signaling pathway is important for regulation of both Th1 and Th2 cytokine gene expression. CTLA-4-mediated reduction of T cell responses may occur via inhibition of either a proximal signal in the CD28/TCR cascade, or of a transcription factor that would be common to all of those cytokines. Although Th2 cells display blunted calcium mobilization (35) and reduced tyrosine phosphorylation of intracellular substrates compared with Th1 cells (36), Erk1 and Erk2, JNK, and p38 mitogen-activated protein kinase (MAPK) are activated similarly by TCR/CD28 ligation in both Th1 and Th2 cells (T. F. Gajewski, unpublished observations). It is conceivable that one of these central signaling pathways shared by Th1 and Th2 cells is a target for CTLA-4 inhibition. This hypothesis is in keeping with a recent report showing that, during TCR stimulation, CTLA-4 cross-linking could selectively reduce the kinase function of Erk and JNK. In contrast, more proximal events, such as CD3-ζ chain phosphorylation and recruitment of ZAP70 to the TCR complex, did not appear to be affected by CTLA-4 ligation (37). CD28 was expressed similarly by the Th1 and Th2 clones used in this study. In addition, although the Th2 clones did not require CD28 stimulation for induction of cytokine secretion, anti-CD28 mAb increased TCR-driven cytokine production in both sets of clones (data not shown). Therefore, our data do not determine whether CTLA-4 inhibits CD28 or TCR-dependent signals in this setting.

We and others have shown that the cytoplasmic tail of CTLA-4 associates with the medium chain of the clathrin adaptor AP-50, which correlates with CTLA-4 endocytosis (22–24). However, it is not known whether CTLA-4 function is performed by the surface or the cytoplasmic pool of the molecule. Cellular distribution was similar in Th1 and Th2 cells and similar to what was observed previously in naive T cells after activation (15). The majority of CTLA-4 was found in intracellular stores rather than on the cell surface. No surface CTLA-4 was detectable in activated Th1 clones. However, a small amount of total CTLA-4 did reach the surface in those cells, as the endocytosis experiments showed accumulation of CTLA-4 in endocytic vesicles in the unpermeabilized Th1 clones, as well as in the Th2 clones, suggesting a similar trafficking pattern in both cell types.

Blocking of CTLA-4 using soluble anti-CTLA-4 mAb only marginally increased Ag-induced T cell proliferation in vitro and did not prolong or postpone peak proliferation. This is in contrast to the continuous lymphoproliferation occurring in CTLA-4-deficient mice in vivo (26, 27) and suggests that factors other than CTLA-4 are responsible for regulation of thymidine incorporation following activation of T cells in vitro. It seems likely that either the decline in Ag concentration or the death of Ag-bearing APCs is responsible for the biphasic proliferation curve observed in vitro.

**FIGURE 7.** Soluble anti-CTLA-4 mAb marginally increases T cell proliferation in both Th1 and Th2 clones. The Th1 clone pGL10 and the Th2 clone pL104 were stimulated with OVA and irradiated syngeneic APCs in the presence of soluble hamster IgG or anti-CTLA-4 mAb. Plates were pulsed for 6 h with [3H]thymidine at the indicated time points after activation, then harvested. Symbols represent the mean ± SD of triplicate determinations and results are representative of three independent experiments.
It is conceivable that persistence of Ag and APCs is required to sustain the lymphoproliferation that occurs in CTLA-4-deficient mice in vivo. Such Ags would be derived either from environmental sources or from self tissues. This hypothesis is in keeping with the observation that interbreeding of CTLA-4-deficient mice onto a TCR-transgenic/RAG2-deficient background abrogates the lymphoproliferation and activated T cell phenotype normally observed in CTLA-4-deficient animals (Ref. 38; M.-L. Alegre, C. B. Thompson, and T. F. Gajewski, unpublished observations). Analysis of the properties of T cells from such mice will allow a determination of the precise role that CTLA-4 plays in regulating T cell expansion.

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FIGURE 8. Cross-linked anti-CTLA-4 mAb decreases the production of cytokines by both Th1 and Th2 clones. Plates were coated overnight with goat anti-hamster IgG at 4°C, followed by anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), and either hamster IgG or anti-CTLA-4 (50 μg/ml) for 2 h at 37°C. The Th1 clone pGL10 and the Th2 clone pL104 were added to the plates and incubated at 37°C. Supernatants were collected at 24 h for detection of IL-2, IL-3, IL-4, IL-5, and IL-10 and at 48 h for analysis of IFN-γ by ELISA. Bars represent the mean ± SD of duplicate determinations, and results are representative of two independent experiments.
FIGURE 9. Cross-linked anti-CTLA-4 mAb decreases cytokine production by bulk populations of Th1 and Th2 cells. Bulk populations of Th1 and Th2 lines were derived as described in Materials and Methods. The experiment was performed as described in Figure 8. Bars represent the mean ± SD of duplicate determinations, and results are representative of two independent experiments.

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References


