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Absence of CTLA-4 Lowers the Activation Threshold of Primed CD8\(^+\) TCR-Transgenic T Cells: Lack of Correlation with Src Homology Domain 2-Containing Protein Tyrosine Phosphatase

Thomas F. Gajewski, Francesca Fallarino, Patrick E. Fields, Fabiola Rivas, and Maria-Luisa Alegre

To examine the role of CTLA-4 in controlling Ag-specific CD8\(^+\) T cell activation, TCR-transgenic/CTLA-4 wild-type or -deficient mice were generated in a recombination-activating gene 2-deficient background. Naive T cells from these mice responded comparably whether or not CTLA-4 was expressed. In contrast, primed T cells responded more vigorously if they lacked CTLA-4 expression. We took advantage of the difference between naive and primed T cell responses to approach the mechanism of CTLA-4 function. Single-cell analyses demonstrated that a greater fraction of CTLA-4-deficient cells responded to a fixed dose of Ag compared to CTLA-4-expressing cells, whereas the magnitude of response per cell was comparable. A shift in the dose-response curve to APCs was also observed such that fewer APCs were required to activate CTLA-4-deficient T cells to produce intracellular IFN-\(\gamma\) and to proliferate. These results suggest that CTLA-4 controls the threshold of productive TCR signaling. Biochemical analysis comparing stimulated naive and primed TCR-transgenic cells revealed no obvious differences in expression of total CTLA-4, tyrosine-phosphorylated CTLA-4, and associated Src homology domain 2-containing protein tyrosine phosphatase. Thus, the biochemical mechanism explaining the differential inhibitory effect of CTLA-4 on naive and primed CD8\(^+\) T cells remains unclear.

Perhaps the best evidence that CTLA-4 is a negative regulator of T cell activation has come from the generation of CTLA-4-deficient mice, which exhibit a profound lymphoproliferative syndrome resulting in death of the animals within 3–5 wk of age (17, 18). However, the activated phenotype of T cells from these mice makes it difficult to study the role of CTLA-4 in controlling the activation of naive T cells. CTLA-4-deficient mice have also been intercrossed with TCR-transgenic mice, but endogenous TCR gene rearrangements generate activated T cells of other specificities that still can mediate autoimmunity and can confound the results being sought regarding naive T cells. In addition, the lymphoproliferation observed in CTLA-4−/− mice is eliminated upon in vivo depletion of CD4+ cells (19), raising questions concerning a potential role for CTLA-4 in modulating the activity of CD8+ T cells.

We sought to examine whether both naive and primed CD8+ cells could be inhibited by CTLA-4, whether CTLA-4 ligation decreased the number of responding cells or the magnitude of response per individual cell, and to seek biochemical evidence in normal T cells supporting the model of CTLA-4 phosphorylation and SHP-2 recruitment in delivery of an inhibitory signal. To this end, CTLA-4-deficient mice were intercrossed with 2C TCR-transgenic mice (kindly provided by Dr. Celeste Simon while at the University of Chicago) to obtain 2C/2C/CTLA-4−/− (21). CTLA-4-deficient mice (generously provided by Dr. Craig Thompson while at the University of Chicago) were generated as described previously (17) and also were bred to homozygosity with Rag2-deficient mice. These were then bred with 2C/2C/− mice to obtain 2C/2C/CTLA-4−/−, 2C/2C/−/−CTLA-4−/−, or 2C/CTLA-4−/−/CTLA-4−/− mice. All transgenic mice were on a C57BL/6 background and were bred in a specific pathogen-free barrier facility at the University of Chicago. Mice were used at 6–8 wk of age for experiments.

Materials and Methods

Mice

2C TCR-transgenic mice were developed as described previously (20) and obtained originally from Dr. Dennis Loh (Washington University, St. Louis, MO). These were intercrossed with Rag2-deficient mice (kindly provided by Dr. Celeste Simon while at the University of Chicago) to obtain 2C/2C/CTLA-4−/− mice (21). CTLA-4-deficient mice (generously provided by Dr. Craig Thompson while at the University of Chicago) were generated as described previously (17) and also were bred to homozygosity with Rag2-deficient mice. These were then bred with 2C/2C/− mice to obtain 2C/2C/CTLA-4−/−, 2C/2C/−/−CTLA-4−/−, or 2C/CTLA-4−/−/CTLA-4−/− mice. All transgenic mice were on a C57BL/6 background and were bred in a specific pathogen-free barrier facility at the University of Chicago. Mice were used at 6–8 wk of age for experiments.

Cell lines and transfectants

The DBA/2-derived mastocytoma P815 was cultured in DMEM supplemented with 10% FCS and incubated at 37°C in an 8% CO2 atmosphere. This P815 line lacks endogenous expression of B7-1 or B7-2, lacks expression of CD40, and fails to stimulate proliferation of purified naive 2C/2C/CTLA-4−/− T cells in vitro. P815.B71 was generated and maintained as described (22). P1.HTR is a highly transfected variant of P815 that grows semidiaphoretically in tissue culture and as a solid tumor mass when implanted s.c. in vivo (23). This cell was used for immunization by in vivo tumor rejection. The C57BL/6-derived thymoma cell line EL4 also was cultured in DMEM with 10% FCS and was used as a syngeneic negative control stimulator cell.

Purification of CD8+ T cells

Splenic CD8+ cells were isolated by negative enrichment using a StemSep magnetic separation system (StemCell Technologies, Vancouver, Canada) according to the manufacturer’s protocol. The purity of the eluted fraction, determined by flow cytometry using the clonotypic Ab 1B2 or anti-CD8 mAb, ranged between 92 and 96%.

T cell thymidine incorporation assay

CD8+ T cells (3 × 10^6) were mixed with 3 × 10^6 mitomycin C-treated P815 or P815.B71 tumor cells in microtiter plates in a final volume of 200 µl. Cultures were pulsed at various times with 1 µCi of [H]Tdr/ml well and harvested 8 h later onto glass filters using a Packard 96-well plate harvester (Packard, Meriden, CT). Incorporation radioactivity was assessed using a Packard Top Count microplate scintillation counter (Packard).

In vivo priming of mice by tumor rejection

2C/2C/CTLA-4−/− or 2C/2C/CTLA-4−/− mice were immunized s.c. in the left flank with 10 × 10^6 living P1.HTR tumor cells in 100 µl of sterile Dulbecco’s PBS. The animals were sacrificed 10 days after immunization, spleen cells were obtained, and CD8+ T cells were purified by negative enrichment, as described above. Following confirmation of purity by flow cytometry, cells were stimulated to assess proliferation and cytokine production.

Abs and flow cytometry

For flow cytometry, PE-coupled anti-CTLA-4 mAb and anti-Thy-1.2 mAb (BD PharMingen, San Diego, CA), FITC-coupled 1B2 (prepared in our laboratory), FITC-coupled anti-CD44 (BD PharMingen), and biotinylated anti-CD28 and anti-CD8 mAbs (BD PharMingen) were used. Expression of the TCR, CD44, CD28, and CD8 was determined by normal surface staining, whereas CTLA-4 expression was assessed after restimulation in vitro (see below) and following saponin permeabilization, as described (6). Nonspecific surface staining was reduced by blocking with the anti-FcγR mAb 2.4G2. Samples were analyzed on a Becton Dickinson FACScan instrument, and data were analyzed using CellQuest software (Becton Dickinson, Mountain View, CA). Live cells were selected for analysis using forward vs side scatter gating.

For CFSE staining, CD8+ T cells were purified by negative enrichment as described above, and 5 × 10^6 cells were washed once and resuspended in Dulbecco’s PBS at a concentration of 2 × 10^7/ml. An equal volume of a CFSE (Molecular Probes, Eugene, OR) solution (5 µM in PBS) was added, and cells were incubated at room temperature for 8 min. The reaction was quenched by the addition of 500 µl of FCS. Cells were washed twice in complete medium, counted, and plated in 24-well plates (Costar, Cambridge, MA) at 1.8 × 10^6/well; each well then received 1.8 × 10^5 mitomycin C-treated P815.B71 cells in a total volume of 1.2 ml of medium. One well was harvested each day, and cells were surface-stained with PE-coupled anti-Thy-1.2 mAb before analysis by flow cytometry.

Stimulation of T cells for lymphoproliferation and CTLA-4 expression

For lymphoproliferation, purified CD8+ T cells (1.8 × 10^5) were stimulated with mitomycin C-treated P815 or P815.B71 tumor cells (1.8 × 10^5) in a total volume of 1.2 ml. Supernatants were collected at 24 h to determine IL-2 production and at 48 h to assess for IFN-γ content. To assess CTLA-4 expression, flow cytometric analysis was performed at the indicated times on permeabilized cells using the anti-CTLA-4 mAb 4F10.

Lymphoproliferative assays and intracellular cytokine measurements

IFN-γ and IL-2 concentrations were measured by ELISA using Ab pairs obtained from BD PharMingen. Concentrations are expressed in U/ml, as determined using the respective recombinant cytokines as standards. Intracellular IFN-γ levels were measured by flow cytometry on permeabilized cells, as described (24).

Protein biochemistry

Naïve or primed 2C/2C/CTLA-4−/− T cells were stimulated for 48 h with P815.B71 cells, as described above, and purified by Ficoll-Hypaque centrifugation. Cells (20 × 10^6) were lysed in a buffer consisting of 0.5% Triton X-100, 50 mM Tris (pH 7.6), 50 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 25 µg/ml p-nitrophenyl p-guanidinobenzoate, and 1 mM PMSF. Lysates remained on ice for 20 min and were then centrifuged at 10,000 × g for 10 min at 14,000 × g. For analysis of whole-cell lysates, supernatants were immediately denatured in reducing sample buffer. For immunoprecipitation of CTLA-4, supernatants were incubated at 4°C for 1 h with protein G-agarose beads that had previously been coated with 4F10 (5 µg/ml). Immunoprecipitating beads were washed with lysis buffer, and the proteins were eluted by boiling in reducing sample buffer. Samples were electrophoresed on 14% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Blots were blocked with 5% BSA, incubated with either a combination of phospho-specific CTLA-4 Abs (1 µg/ml each in 5% milk buffer) (12) or a goat anti-CTLA-4 antisera (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 in TBST), and developed using the appropriate HRP-conjugated second Ab (American Pharmacia Biotech, Piscataway, NJ). SHP-2 was detected using a specific rabbit antiserum (Upstate Biotechnology, Lake Placid, NY). All blots were then stripped and reblotted with an appropriate secondary HRP-conjugated second Ab and developed as above. The signal for SHP-2 was monitored by chemiluminescence, and the protein bands were quantified by densitometry.
Results

Failure to observe augmented responses of naive T cells from 2C/RAG2−/−/CTLA-4−/− compared with CTLA-4+/+ mice

Purified naive 2C/RAG2−/− T cells respond well to P815.B71 cells in vitro, resulting in production of IL-2 and IFN-γ, proliferation, and differentiation into effector CTL (22). Use of the RAG2-deficient background eliminates expression of endogenous TCR genes and ensures a naive T cell surface phenotype (22). 2C/RAG2−/− mice also vigorously reject allogeneic P815 tumors in vivo, resulting in priming of the majority of the T cells in the mouse based on surface expression of CD44 and CD62L, as well as induction of cytolytic activity (21, 25). Thus, immunization via mouse based on surface expression of CD44 and CD62L, as well as flow cytometry. As shown in Fig. 2, the majority of cells from 2C/RAG2−/−/CTLA-4−/− and CTLA-4−/− mice were immunized with living P1.HTR tumor cells in vivo. Splenic CD8+ T cells were isolated 10 days later and analyzed by flow cytometry. As shown in Fig. 2, the majority of cells from 2C/RAG2−/−/CTLA-4−/− and CTLA-4−/− were stimulated without (A) or with (B) P815.B71 cells, and supernatants were collected after 24 h to assess IL-2 production. Similar results were obtained in three independent experiments.

T cell priming yields augmented responsiveness of CTLA-4−/− cells

We reasoned that T cell priming might be necessary to reveal a potential inhibitory effect of CTLA-4. To this end, 2C/RAG2−/−/CTLA-4−/−, CTLA-4−/−, and CTLA-4−/− mice were immunized with living P1.HTR tumor cells in vivo. Splenic CD8+ T cells were isolated 10 days later and analyzed by flow cytometry. As shown in Fig. 2, the majority of cells from 2C/RAG2−/−/CTLA-4−/− and CTLA-4−/− mice were primed as assessed by increased CD44 expression, and comparable levels of clonotypic TCR also were maintained. Similar results were observed for the CTLA-4−/− T cells (data not shown). Each of these cell types was then stimulated with P815 or P815.B71 cells, and thymidine incorporation was measured over time. As shown in Fig. 3, A and B, substantially greater proliferation of the CTLA-4−/− cells compared with the CTLA-4−/− or CTLA-4−/− cells was observed following priming by immunization in vivo. In addition, the difference between cell types was most striking when B7-1 was expressed on the stimulator cells. Thus, B7-1 costimulation resulted in greater augmentation of proliferation of CTLA-4−/−-deficient than of CTLA-4-expressing T cells.

Production of both IL-2 (Fig. 3C) and of IFN-γ (Fig. 3D) by 2C/RAG2−/−/CTLA-4−/− T cells was augmented ~2-fold compared with that seen with the CTLA-4−/− or CTLA-4−/−+ cells. This difference was again seen when P815.B71, but not when P815, cells were used as stimulators. Thus, the increased proliferation of the CTLA-4-deficient T cells can be attributed to greater production of the autocrine growth factor IL-2. Similar results were also seen when the various T cell populations were primed with P815.B71 cells in vitro (data not shown).

Finally, it was conceivable that differences in proliferation between primed CTLA-4−/− and CTLA-4−/− cells were due to less death in the CTLA-4-deficient population. However, this was not the case, as the number of live cells, dead cells, and total cells was increased proportionally in the CTLA-4−/− cell population (data not shown).

Single-cell analysis upon activation of primed CTLA-4−/− and CTLA-4−/− cells

Increased responsiveness of the primed CTLA-4−/− T cell population could result either from a greater number of T cells being

![FIGURE 1](http://www.jimmunol.org/). Thymidine incorporation and IL-2 production by naive CD8+ T cells from 2C/RAG2−/−/CTLA-4−/− wild-type or -deficient mice. A, CD8+ T cells purified by negative selection from naive CTLA-4+/+ or CTLA-4−/− 2C/RAG2−/− mice were stimulated with mitomycin C-treated P815. B71 cells. Replicate cultures were pulsed daily with [3H]Tdr and harvested, and incorporated radioactivity was assessed. B, IL-2 production by naive CD8+ T cells from 2C/RAG2−/−/CTLA-4−/− mice was augmented ~2-fold compared with that seen with the CTLA-4−/− or CTLA-4−/−+ cells. This difference was again seen when P815.B71, but not when P815, cells were used as stimulators. Thus, the increased proliferation of the CTLA-4-deficient T cells can be attributed to greater production of the autocrine growth factor IL-2. Similar results were also seen when the various T cell populations were primed with P815.B71 cells in vitro (data not shown). Finally, it was conceivable that differences in proliferation between primed CTLA-4−/− and CTLA-4−/− cells were due to less death in the CTLA-4-deficient population. However, this was not the case, as the number of live cells, dead cells, and total cells was increased proportionally in the CTLA-4−/− cell population (data not shown). Single-cell analysis upon activation of primed CTLA-4−/− and CTLA-4−/− cells Increased responsiveness of the primed CTLA-4−/− T cell population could result either from a greater number of T cells being
activated or from an increased magnitude of response by individual cells. To address these possibilities, three types of single-cell analyses were performed by flow cytometry. One of the early measurable events that occurs when T cells are stimulated is an increase in cell size. Examination of cell size by forward light scatter following stimulation with P815.B71 cells revealed that a greater proportion of primed 2C/RAG2−/−/CTLA-4−/− T cells was undergoing blastogenesis at 24 h compared with primed CTLA-4−/+ cells (73 vs 30%; Fig. 4A). Even 48 h after activation, a fraction of CTLA-4−/+ T cells had not been triggered to increase in size. In contrast to primed cells, naive CTLA-4−/− T cells did not show this more rapid and uniform cell size increase compared with naive CTLA-4−/+ cells (data not shown).

Inasmuch as blastogenesis precedes mitosis, the above results would predict that a greater fraction of the CTLA-4−/− T cell population should undergo cell division than the CTLA-4−/+ population. This possibility was assessed by labeling the T cells with CFSE and examining loss of fluorescence per cell following activation, the intensity of which is approximately halved with each cell division as the dye is distributed between daughter cells. As shown in Fig. 4B, the rate of cell division for those cells that actually divided was comparable between primed CTLA-4−/+ and CTLA-4−/− T cells, as reflected by the number of cell divisions achieved by 48 h. However, a significant fraction (16%) of the CTLA-4−/+ cells maintained high CFSE staining, reflecting a failure to divide. Gating on this nondividing population revealed that it corresponded to the fraction of cells that had not increased in size by day 2 (data not shown). In contrast to primed cells, naive CTLA-4−/− T cells did not show this increased proportion of cells dividing when compared with naive CTLA-4−/+ cells and, in fact, lagged slightly behind (data not shown), consistent with the modest reduction in thymidine incorporation observed (Fig. 1). Taken together, these results suggest that in the absence of CTLA-4, a greater proportion of the primed T cell population undergoes blastogenesis, DNA synthesis (as measured by thymidine uptake), and cell division, with more rapid kinetics.

In addition to recruiting a higher proportion of T cells to become activated, it was possible that the absence of CTLA-4 also increased the magnitude of response on an individual cell level. This hypothesis was assessed by performing intracellular staining for...
IFN-γ production by flow cytometry at various time points following restimulation of primed T cells in vitro. As shown in Fig. 5A, there was no detectable IFN-γ in T cells isolated from immunized 2C/RAG2−/−/CTLA-4−/− or CTLA-4−/− mice at baseline (time 0). Interestingly, at each of the early time points tested, a greater fraction of CTLA-4-deficient cells was producing IFN-γ compared with the CTLA-4−/− cells (0.9 vs 0.6% at 2 h, 7.2 vs 0.8% at 4 h, and 11.9 vs 2.9% at 7 h). In contrast, the mean fluorescence intensity of the IFN-γ-positive cell fraction was similar between the CTLA-4−/− and the CTLA-4−/− cells at each of the various time points (ranging from 29–36 fluorescence units). Thus, the 2-fold increase in IFN-γ detected in the supernatants of the primed CTLA-4−/− T cell population upon restimulation in vitro (Fig. 3) can be accounted for by an increased number of cells secreting IFN-γ at early time points after activation, rather than by an increased quantity of IFN-γ being produced per cell.

To address directly whether CTLA-4−/− cells displayed a lower activation threshold, graded numbers of P815.B71 cells were used to stimulate primed CTLA-4−/− vs CTLA-4−/− T cells, and intracellular cytokine analysis was performed 6 h later at each stimulator cell dose. As shown in Fig. 5B, a stimulator:responder ratio of 0.1 was required to begin to detect significant numbers (6.6%) of IFN-γ-positive cells in the CTLA-4−/− population. In contrast, ~10-fold fewer stimulator cells (at a ratio of 0.01) were required to elicit IFN-γ staining from a significant fraction (5.8%) of the CTLA-4−/− population. Similar results were observed for intracellular IL-2 staining (data not shown). Thus, absence of CTLA-4 appears to lower the activation threshold of primed CD8+ T cells. In addition, because the increased responsiveness of primed 2C/RAG2−/−/CTLA-4−/− cells was observed only when B7-1 was expressed by the stimulator cells (Fig. 3), this shift in activation threshold is not intrinsic to TCR signaling but appears to depend upon B7-1 ligation of CTLA-4.

Proliferation in response to graded numbers of P815 vs P815.B71 cells was also examined. As shown on Fig. 6, primed 2C/RAG2−/−/CTLA-4−/− and CTLA-4−/− cells proliferated comparably in response to P815 cells (lacking B7 expression), again supporting the notion that TCR signaling was comparable between these T cell populations. However, the CTLA-4−/− T cells demonstrated augmented proliferation in response to P815.B71 stimulator cells, with the dose-response curve shifted substantially to the left. In various experiments, 5- to 100-fold greater P815.B71 cells were necessary to elicit a level of proliferation by CTLA-4−/− T cells that was comparable to that seen by CTLA-4−/− cells. Thus, a shift in activation threshold for proliferation was also observed in the absence of CTLA-4.

Naive and primed 2C/RAG2−/− T cells display comparable expression of CTLA-4, phospho-CTLA-4, and SHP-2 association following activation

It was conceivable that a negative regulatory role for CTLA-4 was manifest only with primed cells because of higher expression of CTLA-4 induced upon activation of primed T cells compared with that induced on naive T cells. However, flow cytometric analysis on permeabilized cells following stimulation in vitro with P815.B71 cells revealed comparable expression of CTLA-4 on both the naive and primed populations, with a similar biphasic kinetics of expression peaking between days 2 and 3 (Fig. 7).

The biochemical mechanism by which CTLA-4 exerts its negative regulatory effect is not completely understood. However, it has been shown that the cytoplasmic tail of CTLA-4 can be phosphorylated by Src family kinases in cotransfection experiments by overexpression (12). This may recruit the interaction of other molecules, including the tyrosine phosphatase SHP-2 (11), which in turn may mediate dephosphorylation of the ζ-chain of the CD3 complex (13). It was conceivable that naive and primed 2C/RAG2−/− T cells differed in the degree of CTLA-4 phosphorylation after stimulation or in expression of (or association with) SHP-2. To examine tyrosine phosphorylation of CTLA-4 with precision, a rabbit antiserum was generated that selectively recognizes tyrosine-phosphorylated, but not nonphosphorylated, CTLA-4, as recently described (12). The specificity of this Ab was verified using Jurkat cells transfected with murine CTLA-4, with or without treatment of the cells with the tyrosine phosphatase inhibitor pervanadate to increase the phosphotyrosine content of intracellular proteins. As shown in Fig. 8A, pervanadate-treated, but not

**FIGURE 4.** A. Analysis of cell size by flow cytometry of primed CTLA-4+/+ vs CTLA-4−/− CD8+ T cells following restimulation in vitro. Purified CD8+ T cells from primed 2C/RAG2−/−/CTLA-4−/− or CTLA-4−/− mice were incubated in vitro in the presence of P815.B71 cells. Cells were collected at each time point, stained with anti-Thy-1.2 mAb and propidium iodide, and analyzed by flow cytometry. Histograms represent the forward light scatter data obtained on 10,000 propidium iodide−/Thy-1.2+ cells. The number in the upper right corner represents the percentage of cells displaying an increased cell size compared with the majority of cells at day 0. Similar results were obtained in two independent experiments. B. Analysis of cell divisions by CFSE-labeling of primed CTLA-4+/+ vs CTLA-4−/−/CD8+ T cells restimulated in vitro. Purified CD8+ T cells from primed 2C/RAG2−/−/CTLA-4−/− or CTLA-4−/− mice were labeled with CFSE and incubated in vitro in the presence of mitomycin C-treated P815.B71 cells. T cells were harvested after 2 days of culture and stained with PE-coupled anti-Thy-1.2 mAb before analysis by flow cytometry. Histograms represent the CFSE fluorescence intensity of 10,000 live Thy-1.2+ cells collected and are representative of two independent experiments.
untreated, Jurkat transfectants contained CTLA-4 that was recognized by this Ab following immunoprecipitation with the anti-murine CTLA-4 mAb 4F10, whereas both conditions yielded comparable amounts of total murine CTLA-4. Western blot analysis using phosphorylated vs nonphosphorylated CTLA-4 peptides confirmed the specificity of this Ab only for phosphorylated CTLA-4 (data not shown). Naive and primed 2C/RAG2−/− T cells were then stimulated for 48 h with P815.B71 cells to induce peak levels of CTLA-4 expression, total CTLA-4 was immunoprecipitated, and Western blot analysis was performed using the anti-phospho CTLA-4 Ab or a goat anti-CTLA4 antiserum. In fact, naive and primed 2C/RAG2−/− T cells showed no obvious differences with respect to expression of phospho-CTLA-4 (Fig. 8B). Thus, the differential regulation of naive and primed 2C T cells by CTLA-4 cannot apparently be explained by gross differences in expression of total CTLA-4, phosphorylated CTLA-4, or levels of expression of SHP-2.

Discussion

Our present results suggest that CTLA-4 exerts an inhibitory effect on the activation of primed rather than of naive CD8+ T cells. Early functional studies using anti-CTLA-4 mAb relied on normal T cells isolated from lymph nodes or the spleen, which are comprised of a mixture of naive and primed T cell populations. It is conceivable that the Ag-experienced cells within those mixtures expressed comparable levels of SHP-2. Immunoprecipitation with anti-CTLA-4 Ab followed by Western blot analysis for SHP-2 also revealed no obvious differences between these cell populations (Fig. 8E). Thus, the differential regulation of naive and primed 2C T cells by CTLA-4 cannot apparently be explained by gross differences in expression of total CTLA-4, phosphorylated CTLA-4, or levels of expression of SHP-2.
Because the present study has used a CD8+ TCR-transgenic mouse model, it does not address directly the potential ability of CTLA-4 to affect naive vs primed CD4+ T cells. It has been reported that the lymphoproliferative syndrome seen in CTLA-4-deficient mice could be eliminated when CD4+ T cells were depleted (19), raising the possibility that, for CD4+ T cells, the naive state may be susceptible to CTLA-4 regulation. Direct examination of CD4+ TCR-transgenic T cells having a naive surface phenotype also revealed modest inhibition by CTLA-4, although primed CD4+ T cells were still inhibited to a greater extent (29). Our current results, along with another recent report (27), do demonstrate that CTLA-4 negatively regulates primed CD8+ T cells. Taken together with the observation that cytokine production by differentiated CD4+ Th1 and Th2 clones is inhibited upon CTLA-4 ligation (10), it seems likely that CTLA-4 does indeed regulate the activation of both CD4+ and CD8+ primed T cells.

T cells have been shown to be capable of expressing B7 family molecules, and it has been suggested that altered glycosylation in T cells makes these ligands unable to bind CD28 while retaining the ability of differentiated effector T cells to continually limit the ability of differentiated effector T cells to continually expand in vivo.

Although CTLA-4-deficient 2C/RAG2−/− T cells displayed increased thymidine incorporation compared with CTLA-4+/+ cells, they did not expand perpetually. Rather, as they reached peak proliferative responses, they subsequently quiesced, and cell death mechanisms appeared to be intact in both CTLA-4−/− and CTLA-4−/− populations. Because primed 2C T cells are potent lytic, it seems likely that the limited proliferative response generally observed in vitro is secondary to elimination of the APCs, thus removing the antigenic stimulus and aborting IL-2 production. That being the case, the lymphoproliferative syndrome seen with conventional CTLA-4-deficient mice in vivo may rely either upon persistence of Ag or upon a survival signal present in vivo but lacking in the in vitro culture.

Cytokine production by T cells appears to be a quantal event, such that when a TCR-mediated activation threshold of a given T cell within a population is crossed, the response from that cell is “all or none” (32). Thus, conditions resulting in increased cytokine production by a given T cell population do so by increasing the fraction of T cells participating in the response. Ligation of CD28 appears to operate, in part, by lowering the activation threshold of T cells, allowing increased numbers of cells to respond to a given TCR stimulus (2). Our present results suggest that engagement of CTLA-4 has the opposite effect, decreasing the fraction of primed T cells among a population that respond, particularly at early time points following stimulation. This observation is consistent with the notion that CTLA-4 engagement raises the activation threshold of cells, counteracting indirectly the positive effect of CD28. This level of control may ensure that T cells bearing the highest affinity TCR, thus having the lowest intrinsic activation threshold, are selected for continued propagation upon repeated encounter with persistent Ag.

Because CTLA-4 can inhibit T cell activation even in the absence of CD28 (25, 33), it seems likely that CTLA-4 ligation actually antagonizes TCR-dependent signaling events. However, the biochemical basis for this inhibition is not completely understood. Cotransfection experiments have demonstrated that Src family kinases can mediate tyrosine phosphorylation of the cytoplasmic tail...
of CTLA-4 (12). Our present results demonstrate for the first time that CTLA-4 also becomes tyrosine-phosphorylated when normal T cells are stimulated with natural ligands on Ag-bearing APCs, supporting the physiologic relevance of this event. Phosphorylation could enable recruitment of molecules that initiate a negative signal capable of counteracting a positive signal delivered via the TCR complex. Alternatively, the rapid endocytosis that CTLA-4 undergoes may mediate sequestration of signaling molecules away from the TCR complex, thus limiting the extent of a positive signal.

The observation that primed T cells are preferentially inhibited through CTLA-4 suggests that biochemical differences in receptor-mediated signaling events exist between naive and previously activated T cells. It is conceivable that CTLA-4 delivers a particular signal only in primed T cells, which allows it to down-regulate T cell activation. Our present results suggest that the functional differences between naive and primed T cells are unlikely to be accounted for by differential phosphorylation of CTLA-4 or by differences in expression of or association with SHP-2. Although our biochemical experiments were not exhaustive and do not rule out the possibility of subtle contributions of these factors, several alternative hypotheses can be considered. It is possible that only a subpopulation of CTLA-4 molecules interacts functionally with the TCR complex to dephosphorylate CD3 components, and that this interaction occurs only in primed T cells. It is also possible that SHP-2 association with CTLA-4 is not sufficient to inhibit TCR signaling. Finally, it is worth considering that the difference in susceptibility to CTLA-4 inhibition between naive and primed T cells may not lie in a difference in CTLA-4 signaling, but rather in a distinction in TCR signaling such that a TCR-activated pathway that is only present in primed T cells is the principal one antagonized by CTLA-4. This possibility would be consistent with the recent observation that activation of the Jnk pathway is detectable in primed, but not naive, T cells (34). A better understanding of the biochemical events triggered by CTLA-4 and by the TCR complex in naive and primed T cells will be necessary to distinguish between these possibilities. Our results suggest that primed TCR-transgenic T cells will provide an ideal model to study signaling via CTLA-4.

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