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CTLA-4 Regulates Tolerance Induction and T Cell Differentiation In Vivo

Theresa L. Walunas and Jeffrey A. Bluestone

Cytotoxic T lymphocyte Ag-4 (CTLA-4; CD152) is an important T cell regulatory molecule. In vitro experiments have shown that the blockade of signals through CTLA-4 augments T cell expansion, while CTLA-4 cross-linking results in decreased T cell proliferation due to decreased IL-2 production. However, less is known about the role of CTLA-4 in regulating an ongoing immune response. In this study, we examined the role of CTLA-4 in the expansion, decline, tolerization, and differentiation of T cells following treatment with staphylococcal enterotoxin B (SEB). Anti-CTLA-4 treatment resulted in increased numbers of SEB-reactive T cells and blockade of subsequent tolerance induction. Further examination of the SEB-reactive cells from anti-CTLA-4-treated mice demonstrated that both the CD4+ and CD8+ Vb8+ T cells produced IL-4, providing evidence that not only do signals through CTLA-4 regulate T cell-tolerizing events, but they also play an important role in the differentiation of T cells in vivo. The Journal of Immunology, 1998, 160: 3855–3860.

CTLA-4 (CD152) is a glycoprotein that is primarily expressed on the surface of activated T cells and shares with CD28 the ability to ligate the B7 family molecules B7-1 (CD80) and B7-2 (CD86; Ref. 1). Current evidence suggests that cross-linking of CTLA-4 has a negative regulatory effect on T cells by down-regulating IL-2 production, cell cycle progression, and anti-CD3-induced cyclins (2–4). Thus, blockade of CTLA-4 signals prolongs T cell activation and expansion both in vitro (5) and in vivo (6, 7). Several additional lines of evidence now exist that suggest that CTLA-4 plays a role in regulating peripheral T cell tolerance and differentiation. First, CTLA-4-deficient mice develop a lymphoproliferative disorder and develop severe autoimmune disease that results in death approximately 3 wk after birth. When restimulated in vitro, T cells from these mice secrete high levels of IL-4 and IFN-γ, consistent with a role for CTLA-4 in the regulation of T cell differentiation (8, 9). In addition, anti-CTLA-4 treatment exacerbates autoimmune disease in a murine model of multiple sclerosis (10). Finally, administration of anti-CTLA-4 mAb to mice concurrently with a tolerizing Ag regimen resulted in the appearance of Ag-reactive T cells (11). Thus, CTLA-4 is likely to play a critical role in the regulation of many aspects of the immune response.

While the systems described above have provided insights into the individual aspects of the immune response, we were interested in examining the role of CTLA-4 throughout the course of an Ag-stimulated immune response. Superantigen administration to mice has been an effective model system in which to study the expansion, decline, and tolerization of Ag-specific T cells. Treatment of mice with staphylococcal enterotoxin B (SEB) results in an initial expansion of Vb8+ T cells that peaks 2 to 3 days following injection. This profound clonal expansion is followed by a precipitous Fas/TNF-regulated (12–14) decline in the number of Vb8+ cells (15). The small number of remaining CD4+Vb8+ T cells are unresponsive to in vitro challenge with SEB or anti-Vb8 mAb as assessed by proliferation and IL-2 production (16, 17). Treatment with whole or Fab fragments of anti-CTLA-4 mAb has been shown to augment the “early” expansion of SEB-reactive T cells (7). However, the role of CTLA-4 in the ultimate outcome of SEB administration, the clonal inactivation of SEB-reactive cells, and the differentiation of these T cells was not addressed.

In this investigation, we directly examined the role of CTLA-4 in the regulation of the superantigen response by studying the effects of anti-CTLA-4 treatment on the early and late stages of SEB-mediated T cell expansion, contraction, inactivation, and differentiation. The results demonstrate that anti-CTLA-4 administration not only results in increased numbers of SEB-reactive T cells in vivo, but enables the SEB-reactive T cells to retain their ability to proliferate and secrete IL-2 when rechallenged with SEB in vitro. Moreover, blockade of CTLA-4 during SEB-mediated Vb8+ activation promoted the differentiation of both CD4+ and CD8+Vb8+ T cell subsets into IL-4-producing T cells. These results support a critical role for CTLA-4 engagement in the regulation of both tolerance induction and T cell differentiation in vivo.

Materials and Methods

Animals

Female BALB/c mice (4–8 wk old) were purchased from Frederick National Cancer Research Institute (Bethesda, MD) and maintained under specific pathogen-free conditions in the University of Chicago Animal Barrier Facility.

Cell lines and reagents

UC10–4F10–11 (4F10) (hamster anti-murine CTLA-4) was produced in an Acucyst Jr. bioreactor (Cellex Biosciences, Minneapolis, MN). The high-titer supernatant was purified over protein A-Sepharose and tested for purity, absence of endotoxin, and functionality as previously described (5). RL172.4 (anti-murine CD4; Ref. 18) ascites and 3.155 (rat anti-murine

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3 Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; Ct Ig, Ig G used as a negative control. Tc, cytotoxic T cell.

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In vivo treatment with SEB

BALB/c mice were treated i.p. with PBS or 100 μg of SEB on day 0. Each day for the next 7 days, animals were treated i.p. with either PBS, 100 μg of Ct Ig, or 100 μg of anti-CTLA-4 mAb as indicated. At the end of treatment, animals were sacrificed and spleen cell suspensions were prepared and analyzed. Splenocytes were depleted of erythrocytes by hypotonic lysis.

Flow cytometry

The number of CD4+ and CD8+ cells expressing the SEB-reactive V8+ TCR and SEB-nonreactive V8+ TCR was determined by two-color flow cytometric analysis. Following harvest, cells were washed once in FACS buffer (1.0% BSA and 0.01% sodium azide in PBS, pH 7.4), incubated with 20 μL 2.4G2 culture supernatant, and simultaneously stained for 30 min at 4°C with FITC-, phycoerythrin- or biotin-conjugated mAb. Cells were then washed in FACS buffer and incubated with phycoerythrin-conjugated streptavidin for 15 min at room temperature. After a final wash, cells were resuspended in FACS buffer and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Incorporation of propidium iodide was used to exclude dead cells. Data from 105 live cells were analyzed using Lysis II software (Becton Dickinson).

Depletion of CD4+ and CD8+ T cells

In each experiment, equal numbers of splenocytes from three SEB-treated and three SEB and anti-CTLA-4-treated mice were individually pooled. Pooled splenocytes were incubated at 2 × 107 cells/ml with a 1:1,000 dilution of anti-CD4 (RL172.4) ascites or a 1:4 dilution of anti-CD8 (3.155) ascites for 30 min at 4°C with FITC-, phycoerythrin- or biotin-conjugated mAb. Cells were then washed in FACS buffer and incubated with phycoerythrin-conjugated streptavidin for 15 min at room temperature. After a final wash, cells were resuspended in FACS buffer and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Incorporation of propidium iodide was used to exclude dead cells. Data from 105 live cells were analyzed using Lysis II software (Becton Dickinson).

Restimulation of SEB-treated cells in vitro

Splenocyte suspensions were normalized for CD3+ or Vγ8+ T cell numbers (as described in Results) and plated at 2 × 106 CD3+ T cells/well or 2.5 × 106 Vγ8+ T cells/well in 96-well round-bottom plates. Cells were cultured with 1 μg/ml SEB, 10 μg/ml SEB, or medium alone (DMEM containing 10% FCS). Supernatants were collected at 24 h to determine IFN-γ levels. Supernatants were prepared in our laboratory. Affinity-purified hamster IgG was used to quantify cytokine levels in the supernatants.

Analysis of cytokine production

Cytokine-containing supernatants were stored at −20°C until analyzed. IL-2 and IL-4 were determined using commercial ELISA kits from Endogen (Cambridge, MA). IFN-γ was determined using an ELISA assay, and reagents were developed and kindly provided by Dr. Robert Schreiber (Washington University, St. Louis, MO). Recombinant murine IL-2, IL-4, and IFN-γ standards were used to quantify cytokine levels in the supernatants.

Results

Anti-CTLA-4 augments expansion of SEB-reactive T cells in vivo

Treatment of mice with SEB results in expansion of Vγ8 T cells. This expansion peaks at 2 to 3 days followed by a precipitous decline in the numbers of SEB-reactive cells (15). Seven days after treatment, the numbers of CD4+Vγ8+ T cells are often below the levels observed before superantigen treatment (23). As seen in Figure 1, the administration of anti-CTLA-4 mAbs, concomitant with injection of SEB, resulted in increased numbers of Vγ8+ T cells compared with that observed in control-treated animals. The increase of Vγ8+ T cells occurs in both the CD4+ (Fig. 1) and CD8+ (data not shown) T cell subsets. Vγ8+ T cell expansion requires antigenic challenge, as anti-CTLA-4 treatment in the absence of SEB treatment did not result in an observable increase in Vγ8+ T cell number (Fig. 1). The effect of anti-CTLA-4 mAbs is specific to SEB-reactive T cell populations, since the absolute numbers of SEB-nonreactive Vγ10+ T cells did not change significantly following superantigen treatment in either the absence or the presence of anti-CTLA-4 mAbs. These results are similar to those of previous studies demonstrating that whole or Fab fragments of anti-CTLA-4 can augment expansion of Ag-reactive (6) and superantigen-reactive (7) T cells. Thus, taken together, these data suggest that blockade of signals through CTLA-4 during a response to SEB blocks a negative signal to activated T cells, resulting in an increase in the number of Ag-reactive T cells.

Blockade of CTLA-4 inhibits tolerance induction by SEB

Previous studies have shown that following in vivo treatment with SEB, the residual CD4+Vγ8+ T cells are hyporesponsive to restimulation with SEB in vitro (16, 17). Figure 2 depicts the in vitro response (normalized for the number of CD3+ T cells) of bulk T cells following PBS or SEB treatment in the presence of control Ig or anti-CTLA-4 mAb therapy. The results showed comparable proliferative responses of bulk T cells from mice treated with anti-CTLA-4 mAbs at the time of SEB therapy (Fig. 2, bottom). Similar results were observed when whole splenocytes were normalized for Vγ8+ T cells and restimulated with SEB in vitro (Fig. 3, top). It has previously been demonstrated that Vγ8+CD8+ T cells do not become hyporesponsive subsequent to SEB treatment and retain the ability to proliferate and produce IFN-γ when restimulated with SEB (24). Thus, it was possible that low levels of IL-2 produced by the CD4+ T cells in the control SEB cultures along with

FIGURE 1. Blockade of CTLA-4 during an SEB-mediated immune response increases the numbers of superantigen-reactive T cells. Mice were immunized with either 100 μl PBS or 100 μg SEB on day 0. To evaluate the effects of blockade of CTLA-4, mice in both of these groups were then treated under control conditions (PBS or Ct Ig) or with anti-CTLA-4 with 100 μg/mouse daily for 7 days. CD4+ T cells were analyzed by two-color flow cytometry for the number of SEB-reactive Vγ8+ T cells (closed bars) and non-SEB-reactive Vγ10+ T cells (open bars). Results are represented as the absolute number of the indicated T cells/spleen, and the SE of the mean is indicated. All groups are representative of four mice, except the group treated with both SEB and anti-CTLA-4, which is representative of six mice.
Blockade of CTLA-4 during an SEB-mediated immune response promotes IL-2 production by SEB-reactive T cells. Mice were treated as described in the legend to Figure 1. Splenic cells were normalized for CD3+ cell number (2 x 10^5 T cells/well), and samples from individual animals were analyzed for IL-2 production (top) and proliferation (bottom) in response to SEB. To analyze proliferation, cells were stimulated for 72 h with medium or 1 μg/ml SEB, and proliferation in medium alone was subtracted from that of SEB-stimulated proliferation. IL-2 was analyzed 24 h after initiation of culture conditions. Each symbol represents the proliferative response of an individual animal in a treatment group. The horizontal line represents the mean of the individual samples. Increased IL-2 production by splenic cells from SEB/anti-CTLA-4-treated mice compared with SEB/Ct Ig-treated mice is significant (p < 0.01).

SEB stimulation was enough to promote the proliferation of the SEB-reactive CD8+ T cells. Consistent with this hypothesis, very little proliferation by the SEB-reactive CD8+ T cells was observed in the absence of CD4+ T cells. However, these cells did produce IFN-γ at levels comparable with that observed from the T cells from control SEB-treated animals, suggesting that they were not unresponsive to restimulation with SEB (data not shown). By comparison, depletion of CD8+ T cells resulted in an increased proliferative response by T cells from mice treated with both SEB and anti-CTLA-4, as compared with T cells from control SEB-treated mice (Fig. 3, bottom). Thus, the CD8+ T cells may consume the increased IL-2 produced by splenic T cells from mice treated with both anti-CTLA-4 mAb and SEB but proliferate less well due to the less effective interaction of SEB with CD8+ T cells, thus reducing the incorporation of [3H]thymidine in the culture (Fig. 2, top). These data suggest that the SEB-reactive CD4+V_{p8}+ T cells were more responsive to Ag following anti-CTLA-4 treatment.

Blockade of CTLA-4 promotes the differentiation of IFN-γ and IL-4-producing T cells

Blockade of CTLA-4/B7 interactions promoted a significant increase in IL-2 production and proliferative responses by SEB-reactive CD4+ T cells. Consistent with this hypothesis, very little proliferation by the SEB-reactive CD8+ T cells was observed in the absence of CD4+ T cells. However, these cells did produce IFN-γ at levels comparable with that observed from the T cells from control SEB-treated animals, suggesting that they were not unresponsive to restimulation with SEB (data not shown). By comparison, depletion of CD8+ T cells resulted in an increased proliferative response by T cells from mice treated with both SEB and anti-CTLA-4, as compared with T cells from control SEB-treated mice (Fig. 3, bottom). Thus, the CD8+ T cells may consume the increased IL-2 produced by splenic T cells from mice treated with both anti-CTLA-4 mAb and SEB but proliferate less well due to the less effective interaction of SEB with CD8+ T cells, thus reducing the incorporation of [3H]thymidine in the culture (Fig. 2, top). These data suggest that the SEB-reactive CD4+V_{p8}+ T cells were more responsive to Ag following anti-CTLA-4 treatment.

Enhanced IL-4 production is observed from both CD4+ and CD8+ T cell subsets

The previous results could not distinguish whether anti-CTLA-4 treatment was promoting the expansion of Th0- or both Th1- and
Th2-type T cells. In addition, the presence of both CD4\(^+\) and CD8\(^+\) T cells further complicated the analyses, since studies by other investigators have shown that CD8\(^+\) T cells isolated from SEB-treated mice retain their ability to secrete IFN-\(\gamma\) when re-stimulated with SEB in vitro (24). Therefore, cytokine production by the individual CD4\(^+\) and CD8\(^+\) T cell subsets was examined to determine the source of IL-4 and IFN-\(\gamma\). As seen in Figure 5, right, depletion of CD8\(^+\) T cells resulted in significantly diminished levels of IFN-\(\gamma\) production by the residual CD4\(^+\) T cells in response to SEB. Thus, the majority of the increase in IFN-\(\gamma\) levels can be attributed to the expanded CD8\(^+\) T cell subset. In contrast, depletion of CD8\(^+\) T cells reduced, but did not eliminate, IL-4 production. These results suggested that CTLA-4 blockade during a response to SEB promoted the differentiation of the CD4\(^+\) V\(_\beta\)8\(^+\) T cells into IL-4-producing Th2-type T cells. Interestingly, CD8\(^+\) T cells isolated from SEB- and control-treated mice produced little, if any, IL-4 (Fig. 4, left). In contrast, at least a small subset of CD8\(^+\) T cells from anti-CTLA-4-treated mice were observed to produce IL-4 (Fig. 5, left), suggesting that the blockade of CTLA-4 enhanced IL-4 production by CD8\(^+\) T cells as well. However, it remains to be clarified as to whether the IL-4 and IFN-\(\gamma\) are being produced by a cytotoxic T cell (Tc) 0-like population or a mixture of Tc1- and Tc2-like differentiated cells.

**Discussion**

The regulatory role of CTLA-4 during an immune response is beginning to be understood. Although some studies have suggested a costimulatory role for this molecule (27), our studies, along with several others, have implicated CTLA-4 in the attenuation of T cell expansion. Blockade of CTLA-4/B7 interactions in vitro or in vivo with Abs, or via deletion of the CTLA-4 gene, promotes T cell proliferation and cytokine production (2–9). In the present studies, we have shown that injection of blocking anti-CTLA-4 mAbs in mice resulted in IL-4-producing SEB-reactive T cells. Mice were treated as described in the legend to Figure 1. Splenic cells were normalized for CD3\(^+\) T cell number (2 \(\times\) 10\(^5\) T cells/well) and stimulated for 48 h with 10 \(\mu\)g/ml SEB. Samples were then analyzed for IL-4 (left) or IFN-\(\gamma\) (right) by ELISA. Each symbol represents the level of cytokine produced by an individual animal in response to the stimulus. The horizontal line represents the mean of the individual samples. IL-4 produced by PBS- or SEB-treated mice treated with anti-CTLA-4 is significantly increased over that observed from control-treated mice (p < 0.01). IFN-\(\gamma\) produced by SEB/anti-CTLA-4-treated mice is significantly increased over that observed by SEB/Ct Ig-treated mice (p < 0.05).

**FIGURE 4.** Anti-CTLA-4 treatment during an immune response to SEB results in IL-4-producing SEB-reactive T cells. Mice were treated as described in the legend to Figure 1. Splenic cells were normalized for CD3\(^+\) T cell number (2 \(\times\) 10\(^5\) T cells/well) and stimulated for 48 h with 10 \(\mu\)g/ml SEB. Samples were then analyzed for IL-4 (left) or IFN-\(\gamma\) (right) by ELISA. Each symbol represents the level of cytokine produced by an individual animal in response to the stimulus. The horizontal line represents the mean of the individual samples. IL-4 produced by PBS- or SEB-treated mice treated with anti-CTLA-4 is significantly increased over that observed from control-treated mice (p < 0.01). IFN-\(\gamma\) produced by SEB/anti-CTLA-4-treated mice is significantly increased over that observed by SEB/Ct Ig-treated mice (p < 0.05).

**FIGURE 5.** IL-4 is produced by SEB-reactive CD4\(^+\) and CD8\(^+\) T cells. CD4\(^+\) and CD8\(^+\) T cells from mice treated with SEB and anti-CTLA-4 were examined for the production of IL-4 (left) and IFN-\(\gamma\) (right) following stimulation with SEB. CD4\(^+\) (CD8 Depl.) or CD8\(^+\) (CD4 Depl.) T cells were depleted from the pooled spleens of three mice using anti-CD4 or anti-CD8 mAb and complement. Cells were normalized for CD3\(^+\) T cell number (2 \(\times\) 10\(^5\) T cells/well) and stimulated with 10 \(\mu\)g/ml SEB. Production of IL-4 and IFN-\(\gamma\) was evaluated 48 h following stimulation by ELISA. Cytokine production by the bulk pool is determined by averaging the cytokine production of splenic T cells from the individual mice in the pool. Data are representative of two experiments.
the data suggest that CTLA-4 ligation inhibits IL-4 production by both CD4+ and CD8+ T cells.

Other investigators have addressed the roles of CD28 and CTLA-4 in the regulation of SEB-induced tolerance. For instance, expression of a soluble form of CTLA-4 (CTLA4Ig) prevents the induction of T cell unresponsiveness following SEB treatment of mice transgenic for CTLA4Ig (28). These results suggested that CTLA4Ig prevents tolerance by disrupting CTLA-4/B7 interactions. However, CTLA4Ig also blocks CD28 engagement, thus preventing the initial activation event that may be required for tolerance induction. The data shown in the present study provide direct evidence that blockade of CTLA-4 prevents T cell anergy in response to SEB, supporting a role for CTLA-4 in SEB-induced unresponsiveness.

It is unclear by what mechanism CTLA-4 regulates peripheral tolerance. It is possible that the blockade of CTLA-4 signals could either prevent the entry of cells into an anergic state or that mAb treatment might reverse an established anergic state. Several results support the possibility that CTLA-4 blockade allows the Vα8+ T cells to “grow” out of anergy. First, blockade of CTLA-4 signals enhances growth factor production, in particular, IL-2 (10). The addition of exogenous IL-2 has been shown to reverse anergy in Th1 T cell clones in vitro (29), and continuous IL-2 administration during a response to staphylococcal enterotoxin prevents tolerance in the staphylococcal enterotoxin-reactive T cells (30). In addition, blockade of CTLA-4/B7 interactions promotes increased IL-2 expression in vivo (31). CTLA-4 blockade of activated T cells 48 h after SEB immunization, and these cells are more IL-2 responsive in vitro compared with cells from control-treated mice (data not shown). Second, previous studies have shown that CTLA-4 blockade can promote cell cycle progression directly, while CTLA-4 cross-linking inhibits the induction of cyclins (4). This results in increased expansion of activated T cells in vitro (2, 3) and may allow the SEB-reactive cells to overcome unresponsiveness in vivo. Finally, CTLA-4 may play a direct role in maintaining Ag-induced tolerance. Previous studies have shown that blockade of CTLA-4 signals can exacerbate autoimmune disease (10), CTLA-4-deficient mice die from an autoimmune disease (8, 9), and anti-CTLA-4 can block tolerization of Ag-specific T cells when Ag is administered under tolerizing conditions (11).

The studies reported here demonstrate an important role for CTLA-4 in T cell differentiation. Both CD4+ and CD8+ T cells from SEB and anti-CTLA-4-treated mice secrete IL-4 when restimulated with SEB. CTLA-4 blockade might promote IL-4 production in several ways. It has been shown, in vitro, that increasing TCR (31) or CD28 (25, 26) signals promotes the development of Th2-like T cells. These data have led to a hypothesis that development of IL-4-producing T cells is dependent on the “strength of signal” delivered to the T cell through the TCR and CD28. Weaker costimulation promotes development of Th1 cells, while stronger stimuli promote the development of Th2 cells (32, 33). Blockade of CTLA-4 signals may increase the strength of signals delivered to the T cell in several ways. First, extracellularly, CTLA-4 may compete with CD28 for the costimulatory ligands B7-1 and B7-2. Treatment with anti-CTLA-4 mAb may block the interaction of CTLA-4 with these ligands, “freeing up” more of these molecules to interact with CD28, increasing the signals through CD28, and thereby promoting IL-4 production. Second, CTLA-4 may compete with CD28 at the intracytoplasmic level. Both CD28 and CTLA-4 have cytoplasmic SH2 domains that can interact with phosphatidylinositol 3-kinase (34). CTLA-4 signal blockade may provide CD28 with an increased pool of signaling molecules that allow increased signaling through CD28. Finally, CTLA-4 may negatively regulate TCR/CD28 signals directly, CTLA-4 interacts with a tyrosine phosphatase, PTP-1D (35). Translocation of CTLA-4/PTP-1D into the TCR/CD28 complex during an immune response may attenuate signals through the TCR/CD28 by dephosphorylating membrane proximal signaling molecules. Anti-CTLA-4 treatment may prevent CTLA-4 translocation into the TCR/CD28 complex, resulting in increased signaling through these activating components.

Taken together, these studies suggest that CTLA-4 may play an important role in the response to bacterial pathogens where superantigen responses may be a critical component of the immune response. Manipulation of CTLA-4 signals with mAb may provide an important tool for enhancing the host immune response in clinical settings.

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References