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

Theresa L. Walunas and Jeffrey A. Bluestone

*J Immunol* 1998; 160:3855-3860; 
http://www.jimmunol.org/content/160/8/3855

References

This article cites 34 articles, 21 of which you can access for free at: http://www.jimmunol.org/content/160/8/3855.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CTL A-4 Regulates Tolerance Induction and T Cell Differentiation In Vivo1

Theresa L. Walunas and Jeffrey A. Bluestone2

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+ V8+ 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.

1 This research was supported by National Institutes of Health Grants P01 AI35294 and CA14599 and Genetics Institute (Cambridge, MA). T.L.W. was supported by 18 U.S.C. Section 1734 solely to indicate this fact.

2 Address correspondence and reprint requests to Dr. Jeffrey A. Bluestone, 5841 South Maryland Avenue, MC1089, Chicago, IL 60637. E-mail address: jbluest@immunology.uchicago.edu

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Copyright © 1998 by The American Association of Immunologists

Received for publication October 14, 1997. Accepted for publication December 22, 1997.

The Committee on Immunology and the Ben May Institute for Cancer Research, The University of Chicago, Chicago, IL 60637

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-titered supernant 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 CD3, 16A3) were obtained from the American Type Culture Collection (Rockville, MD). 3.155 was used as a negative control. Tc, cytotoxic T cell.

Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; Ct Ig, Ig G used as a negative control. Tc, cytotoxic T cell.
CD8; Ref. 19) and 2.4G2 (rat anti-murine FeR; Ref. 20) culture superna-
tants were prepared in our laboratory. Affinity-purified hamster IgG was
 purchased from Cappel Research Products (Durham, NC) and was used as
 a negative control (Ct Ig) for 4F10. FITC-conjugated anti-CD8 (YTS
 169.42; Ref. 21) and rat anti-murine CD4 (GK1.5; Ref. 22) were prepared
 in our laboratory. FITC-conjugated 145-2C11 (hamster anti-murine CD3e)
 was provided by Boehringer Mannheim (Indianapolis, IN). Biotin-conju-
gated anti-VEesper8.1/Vesper8.2 (MR5-2) and phycoerythrin-conjugated anti-VEesper10
 (B21.5) were purchased from PharMingen (San Diego, CA). Phyco-
erthrin-conjugated streptavidin was purchased from Southern Biotechnol-
 ogy Associates (Birmingham, AL). SEB was purchased from Sigma Chem-
 ical (St. Louis, MO).

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 hypo-
tonic 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 mAbs. 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 io-
dide was used to exclude dead cells. Data from 10⁴ 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 × 10⁶ cells/ml with a 1:1000 dilu-
tion of anti-CD4 (RL172.4) ascites or a 1:4 dilution of anti-CD8 (3.155)
 mAb. Pooled splenocytes were incubated at 2 × 10⁶ cells/ml with a 1:1000 dilu-
tion of anti-CD4 (RL172.4) ascites or a 1:4 dilution of anti-CD8 (3.155)
 culture supernatant for 30 min at 4°C. An equal volume of rabbit comple-
 ment (Pel-Freez, Brown Deer, WI), diluted 1:10 in 4°C PBS, was added to
 the cells, and the cells were incubated at 37°C for 45 min with constant
 mixing. Cells were passed over Ficoll-Hypaque to eliminate dead cells.
 Efficiency of depletion was determined by flow cytometry to be greater
 than 95%.

Restimulation of SEB-treated cells in vitro
Splenocyte suspensions were normalized for CD3⁺ or V₈⁺ T cell num-
 bers (as described in Results) and plated at 2 × 10⁵ CD3⁺ T cells/well or
 2.5 × 10⁵ V₈⁺ 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
 IL-2 production and at 48 h to determine IFN-γ and IL-4 production. Cells
 were harvested at approximately 64 h after culture initiation following a
 16-h pulse with 1 μCi/well of [³H]thymidine (Amersham, Arlington
 Heights, IL) to assess proliferation. Data points are represented as the mean
 of triplicate wells. SEs were less than 20%.

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 Endo-
gen (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 V₈⁺ 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⁺V₈⁺ 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 V₈⁺ T cells
 compared with that observed in control-treated animals. The increase of V₈⁺ T cells occurs in both the CD4⁺ (Fig. 1) and
 CD8⁺ (data not shown) T cell subsets. V₈⁺ T cell expansion requires antigenic challenge, as anti-CTLA-4 treatment in the ab-
 sence of SEB treatment did not result in an observable increase in
 V₈⁺ 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 V₈⁺ T cells did not change signi-
 ficantly following superantigen treatment in either the absence
 or the presence of anti-CTLA-4 mAb. These results are similar to
 those of previous studies demonstrating that whole or Fab frag-
 ments 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⁺V₈⁺ T cells are hyporesponsive to re-
stimulation 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 pro-
liferative 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 V₈⁺ T cells and restimulated with SEB in vitro (Fig. 3, top).
 It has previously been demonstrated that V₈⁺CD8⁺ T cells do not
 become hyporesponsive subsequent to SEB treatment and re-
tain the ability to proliferate and produce IFN-γ when restimulated
 with SEB (24). Thus, it was possible that low levels of IL-2 pro-
duced by the CD4⁺ T cells in the control SEB cultures along with

Figure 1. Blockade of CTLA-4 during an SEB-mediated immune re-
sponse increases the numbers of superantigen-reactive T cells. Mice were
 immunized with either 100 μg 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 V₈⁺ T cells (closed bars)
 and non-SEB-reactive V₈⁺ 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.
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-\(\gamma\) 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. To analyze proliferation, cells were stimulated for 72 h with medium or 1 \(\mu\)g/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 \((p < 0.01)\).

Blockade of CTLA-4 during an SEB-mediated immune response promotes increased proliferation by CD4\(^+\) V\(_b\)8\(^+\) T cells. Mice were treated as described in the legend to Figure 1. Splenic cells were normalized for CD3\(^+\) cell number \((2 \times 10^5\) 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 \(\mu\)g/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 \((p < 0.01)\).

**FIGURE 3.** Blockade of CTLA-4 during an SEB-mediated immune response promotes increased proliferation by CD4\(^+\) V\(_b\)8\(^+\) T cells. Mice were treated as described in the legend to Figure 1. Splenic cells were normalized for V\(_b\)8\(^+\) T cell number \((2.5 \times 10^5\) V\(_b\)8\(^+\) cells/well\), and samples were analyzed for proliferation in response to 1 \(\mu\)g/ml SEB. Top, Bars represent the mean proliferative responses of three individual animals. Bottom, CD8\(^+\) T cells were depleted from splenocytes pooled equally from three mice and restimulated with SEB. Bars represent the proliferative response of these cells. Data are representative of two separate experiments.

T cells. In previous studies, changes in signaling through CD28 have been shown to have effects on T cell differentiation (25, 26). Thus, it was of interest to determine whether changes in CTLA-4 signaling could also affect T cell differentiation. In Figure 4, splenic T cells from mice stimulated in vivo with either PBS or SEB in the presence of control Ig or anti-CTLA-4 mAb treatment were examined for IL-4 \((Fig. 4, left)\) or IFN-\(\gamma\) \((Fig. 4, right)\) production following restimulation in vitro with SEB. T cells from anti-CTLA-4-treated mice produced significantly more IL-4 when restimulated with SEB, a result that was observed regardless of whether the cells were normalized for CD3\(^+\) \((Fig. 4)\) or V\(_b\)8\(^+\) \(T\) cells \((data not shown)\). In addition, IFN-\(\gamma\) production was also augmented by T cells from the anti-CTLA-4/SEB-treated group. These data suggest that blockade of CTLA-4 signals during a response to SEB promotes both IFN-\(\gamma\) and IL-4 production by SEB-reactive T cells. Interestingly, there was a small, but significant, increase in IL-4 production by T cells from mice treated with anti-CTLA-4 alone. In addition, there was a comparable decrease in IFN-\(\gamma\) production. These results may reflect an effect of CTLA-4 blockade on a small number of V\(_b\)8\(^+\) T cells stimulated by endogenous or environmental Ags encountered during the time of treatment with anti-CTLA-4 mAb.

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-γ 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-γ. As seen in Figure 5, right, depletion of CD8^+ T cells resulted in significantly diminished levels of IFN-γ production by the residual CD4^+ T cells in response to SEB. Thus, the majority of the increase in IFN-γ 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_β8^+ 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-γ 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 coinjected with Ag increased the number of surviving T cells in the spleen of SEB-treated mice as compared with mice treated under control conditions. These results are consistent with studies demonstrating enhanced T cell expansion in response to Ag in mice coinfected with anti-CTLA-4 mAbs (6, 7). These studies also show that CTLA-4 engagement blocks proliferation and plays an important role in subsequent inactivation of SEB-exposed T cells. Finally,
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-2Rα expression on in vivo SEB-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 expression 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 re-stimulated 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.

Acknowledgments

We thank Ms. Julie Auger and Mr. Paul Butterfield for flow cytometry assistance.

References


