CTLA-4 Overexpression Inhibits T Cell Responses through a CD28-B7-Dependent Mechanism

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CTLA-4 Overexpression Inhibits T Cell Responses through a CD28-B7-Dependent Mechanism

John J. Engelhardt, ** Timothy J. Sullivan, † and James P. Allison **

CTLA-4 has been shown to be an important negative regulator of T cell activation. To better understand its inhibitory action, we constructed CTLA-4 transgenic mice that display constitutive cell surface expression of CTLA-4 on CD4 and CD8 T cells. In both in vivo and in vitro T cell responses, CTLA-4 overexpression inhibits T cell activation. This inhibition is dependent on B7 and CD28, suggesting that overexpressed CTLA-4 inhibits responses by competing with CD28 for B7 binding or by interfering with CD28 signaling. In addition, expression of the transgene decreases the number of CD25^+Foxp3^+ T cells in these mice, but does not affect their suppressive ability. Our data confirm the activity of CTLA-4 as a negative regulator of T cell activation and that its action may be by multiple mechanisms. *The Journal of Immunology*, 2006, 177: 1052–1061.

**Materials and Methods**

Media and cell culture

All cell culture was performed in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin (all from BioWhittaker), and 2 μM 2-ME (Sigma-Aldrich). All cells were cultured in a 37°C humidified incubator at 5% CO₂, CHO cells expressing I-E^k were obtained from Dr. N. Shastri (University of California, Berkeley, CA), and CA36.1 L cells expressing I-E^k and B7.1 were described earlier.
Mice
CTLA-4 transgenic mice were generated by injection of the CTLA-4 transgenic construct into (C57BL/6 × CBA/J)F1, fertilized eggs. Mice were backcrossed at least 10 times onto the C57BL/6 background for additional experiments. 5C.C7 TCR transgenic mice (RAG2−/−) were obtained from Taconic Farms. CTLA-4 transgenic mice were backcrossed onto the 5C.C7 TCR transgenic RAG2−/− background by breeding onto the 5C.C7 background and backcrossed six times. CD28−/− RAG2−/− 5C.C7 TCR transgenic mice were described earlier, and RAG2−/− CD28−/− 5C.C7 transgenic mice were obtained by crossing 5C.C7 TCR transgenic mice with RAG2−/− mice. All mice were bred and maintained in microisolator cages in accordance with the animal care and use regulations of University of California (Berkeley, CA) and Memorial Sloan-Kettering Cancer Center.

Abs and flow cytometry
The following Abs were used for flow cytometry: TriColor anti-CD4 (RM4-5), Alexa-488 anti-CD8a (53-6-7) (Caltag), PE anti-CTLA-4 (UC10-4F10), PE anti-CD8a, FITC anti-CD69 (H1.2F3), FITC anti-CD62L (MEL-14), biotin anti-CTLA-4 (37.51), PE anti-CD25 (PC61), PerCP anti-CD69 (53-6-7) (BD Pharmingen). PE anti-Foxp3 (FJK-161), FITC anti-CD122, FITC anti-CD24 (IM7), allophycocyanin anti-CD4 (L3T4), and allophycocyanin anti-CD25 (PC61) (eBioscience). For surface staining of lymphocytes or thymocytes, cells were incubated with anti-FcR Ab (2.4G2) and stained with the appropriate Abs in PBS/2% FCS. To perform intracellular staining, cells were fixed with 1% paraformaldehyde/PBS, washed with PBS/2% FCS with 0.3% saponin, and incubated with appropriate Abs; for Foxp3 staining, cells were also blocked with 4% normal rat serum after permeabilization.

T cell assays
Lymph node cells from 5C.C7, 5C.C7 CTLA-4 transgenic, 5C.C7 CD28−/−, and 5C.C7 CD28−/− CTLA-4 transgenic mice were isolated and were ≥90% Vα1/Vγ4 positive. A total of 50,000 lymph node cells was mixed with either 100,000 spleen cells from B10.A mice, or 25,000 L cells expressing I-Ek and B7.1, or 25,000 CHO cells expressing I-Ek and the indoleamine 2,3-dioxygenase. The overexpression of CTLA-4 on naive T cells of an intact animal increased on the surface of CD4+ lymphocytes after they have left the thymus. The overexpression of CTLA-4 in the periphery after they have left the thymus. The CTLA-4 transgenic mice display no gross differences in thymic or peripheral CD4+ or CD8+ T cell populations, and surface expression of CTLA-4 is not evident on transgenic CD4+ CD8− or CD4+ CD8− thymocytes (data not shown). However, the transgene does direct intracellular CTLA-4 expression to thymocytes, which do not normally express CTLA-4 (Fig. 1C). Because CTLA-4 and CD28 both compete for the same ligands, we reasoned that constitutive overexpression of CTLA-4 might affect surface levels of CD28. To test this hypothesis, thymocytes from CTLA-4 transgenic and littermate controls were stained for CD28. We found CD28 to be significantly up-regulated in the CD4 single-positive population, but not in the CD4 CD8− or CD4−CD8+ thymocytes (data not shown).

Suppressor assay
Lymph node and splenic cells from CTLA-4 transgenic mice and littermate controls were isolated and subjected to magnetic bead purification using the Miltenyi CD4+ CD25+ T cell purification kit. Effector cells were isolated and ≥85% CD4+ positive, and CD25 regulatory cells were isolated and >85% CD4 CD25+ positive. Spleen cells were depleted of T cells using pan-T Cell Dynabeads and were irradiated with 2000 rad. A total of 50,000 CD4+ effector cells were mixed with 100,000 T-depleted splenocytes and 5 mg/ml anti-CD3 Ab. CD4+ CD25− T cells were added as indicated. Cells were pulsed with 1 μCi of [3H]thymidine at 64 h and were harvested 8 h later.

Statistical analysis
Statistical analysis was performed using Prism GraphPad software. The Mann-Whitney nonparametric t test was used to calculate p values for mean clinical score data and the individual data points in the EAE graph.

Results
Generation of CTLA-4 transgenic mice
To investigate the role of CTLA-4 in regulating naive T cell responses, we generated transgenic animals overexpressing full-length CTLA-4 under the control of the murine H-2Kb promoter and the 3′ Ig enhancer (Fig. 1A), a combination previously shown to direct expression primarily to the lymphoid lineage (20). Flow cytometric analysis of the CTLA-4 transgenic line with an anti-CTLA-4 Ab demonstrated increased expression of CTLA-4 on both the surface of naive T cells and in the cytoplasm. This increased surface expression is apparent in splenic (data not shown) and lymph node T cell populations (Fig. 1B). These animals provide a useful tool for studying the consequence of CTLA-4 overexpression on T cell development, homeostasis, and activation.

Effect of CTLA-4 overexpression on T cell development
CTLA-4 transgenic mice display no gross differences in thymic or peripheral CD4+ or CD8+ T cell populations, and surface expression of CTLA-4 is not evident on transgenic CD4+ CD8− or CD4+ CD8− thymocytes (data not shown). However, the transgene does direct intracellular CTLA-4 expression to thymocytes, which do not normally express CTLA-4 (Fig. 1C). Because CTLA-4 and CD28 both compete for the same ligands, we reasoned that constitutive overexpression of CTLA-4 might affect surface levels of CD28. To test this hypothesis, thymocytes from CTLA-4 transgenics and littermate controls were stained for CD28. We found CD28 to be significantly up-regulated in the CD4 single-positive population, but not in the CD4 CD8− or CD4−CD8+ populations (Fig. 2A). Levels of CD28 were also found to be significantly increased on the surface of CD4+ lymph node T cells derived from CTLA-4 transgenic animals (Fig. 2B). There are at least two possible reasons for this increase in CD28 expression. First, the CTLA-4 transgene could have an effect on thymocyte development. In this case, higher CD28 levels may be needed for these T cells to successfully pass through positive selection, so CD28 levels increase as a compensatory mechanism. Alternatively, higher CD28 levels may be needed for survival of naive CTLA-4 transgenic T cells in the periphery after they have left the thymus. The up-regulation of CD28 in thymocytes suggests that CD28 expression is up-regulated before reaching the periphery, but does not rule out that it may also be needed in the periphery.

CTLA-4 overexpression alters CD4+ lymph node T cell activation
The overexpression of CTLA-4 on naive T cells of an intact animal may influence the activation of these cells to naturally occurring Ags. To determine whether CTLA-4 overexpression influences the activation of circulating CD4+ T cells, we stained lymph node T cells for the activation markers CD69, CD44, and CD62L. We found that fewer numbers of CD4+ T cells within the CTLA-4 transgenic mice had an activated phenotype (CD69high, CD44high, CD62Llow) compared with littermate control animals (Fig. 2C and Table I). This finding suggests that CTLA-4 overexpression is effectively raising the threshold for activation of these T cells, or limiting their expansion after Ag exposure, and demonstrates that CTLA-4 overexpression inhibits CD4+ T cell activation in vivo.

In contrast with CD4+ T cells, there was no difference in the expression of the activation markers CD44, CD62L, CD69, or

3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MCC, moth cytochrome c; IDO, indoleamine 2,3-dioxygenase.
CD122 on CD8+ T cells (data not shown). This suggests that CTLA-4 overexpression has a minimal effect on the activation or homeostasis of naive CD8+ T cells. Previous studies have demonstrated that TCR transgenic CTLA-4−/− CD8+ T cells do not show increased primary responses to Ag compared with wild-type TCR transgenic CD8+ T cells (21). Both results suggest that CTLA-4 has a lesser effect on the activation of naive CD8+ T cells compared with CD4+ T cells.

Effect of CTLA-4 overexpression on regulatory T cells

Because it is highly expressed on CD25+ T cells, it has been suggested that CTLA-4 plays an important role in their function (22, 23). In previous reports, CD28 costimulation has been reported to be important for the development of CD25+ regulatory T cells. CD28 signaling in the thymus is involved in induction of Foxp3 expression (24), a transcription factor important for regulatory cell function (25–27). It has also previously been reported that CD28−/− mice have reduced numbers of CD4+CD25+ regulatory T cells (28, 29). Because CTLA-4 is expressed in the thymus in CTLA-4 transgenic mice, this may inhibit CD28 signaling needed for the development of regulatory cells. To determine the effect of CTLA-4 overexpression on the development or maintenance of regulatory T cells, lymph node cells were stained for CD4, CD25, and Foxp3. CTLA-4 transgenic mice were found to have approximately one-third the number of CD25+CD4+ T cells or CD4+Foxp3+ cells compared with littermate controls (Fig. 3, A and B). We also found a reduction in the percentage of CD4 single-positive thymocytes expressing Foxp3 and CD25 (Fig. 3C). These findings suggest that CTLA-4 overexpression inhibits the development of regulatory cells, but do not exclude an effect on homeostasis of regulatory T cells in the periphery.

We analyzed CTLA-4 expression levels on CD25+ cells from the lymph node of transgenic and littermate control mice and found that, similar to naive effector cells, CTLA-4 transgenic CD25+ cells expressed higher levels of CTLA-4 (data not shown). If CTLA-4 is indeed important in the suppressive function of regulatory cells, it may be expected that cells that express higher levels of CTLA-4 would also have increased suppressive abilities...
on a per-cell basis. To test this hypothesis, in vitro suppression assays were performed using wild-type and CTLA-4 transgenic CD25+ regulatory T cells. CTLA-4 transgenic CD25+ regulatory T cells were found to inhibit the proliferation of wild-type effector CD4+ T cells to the same extent as wild-type CD25+ regulatory cells (Fig. 3D). These results are consistent with a previous study performed on CTLA-4 transgenic regulatory cells (30). Previous studies have also shown that regulatory cells from CTLA-4-deficient mice retain regulatory activity (30–32). These data do not rule out a role for CTLA-4 in the function of regulatory cells, but
do suggest that the level of CTLA-4 expression is not limiting for regulatory cell function.

**CTLA-4 overexpression results in diminished Ag-specific responses in vitro**

CTLA-4−/− TCR transgenic CD4+ T cells exhibit increased Ag-specific T cell responses compared with wild-type TCR transgenic T cells (33). Thus, we reasoned that overexpression of CTLA-4 would restrict Ag-specific T cell responses. CTLA-4 transgenic animals were crossed to a Rag2-deficient 5C.C7 TCR transgenic background. 5C.C7 T cells recognize aa 88–103 of moth cytochrome c (MCC) presented in the context of I-Ek. T cells from wild-type 5C.C7 and 5C.C7 CTLA-4 transgenic animals were compared in an in vitro T cell proliferation assay using L cells expressing I-Ek and B7.1 or irradiated syngeneic spleen cells as APCs. CTLA-4 transgenic T cells were found to be less responsive to Ag stimulation compared with their littermate control counterparts. Transgenic T cells had a smaller magnitude of response and showed a higher threshold of response to Ag (Fig. 4A). When stimulated with splenic cells, transgenic T cells had a lower magnitude of response and showed a higher threshold of response to Ag (Fig. 4B). In both cases, the lower magnitude of response, and shift in the dose-response curve was regardless of which ligand was expressed on the APC used, because the L cells express B7.1, whereas spleen cells predominately express B7.2. These findings demonstrate that CTLA-4 overexpression can inhibit Ag-specific responses of naive T cells.

CTLA-4 transgenic T cells could be less responsive to Ag for two different reasons. Overexpression of CTLA-4 could interfere with the activation of these T cells, or the T cells that develop in these mice might be inherently less responsive to Ag. Recent data suggesting that a ligand-independent CTLA-4 can inhibit T cell activation (34, 35) imply that overexpression of full-length CTLA-4 may inhibit activation even in the absence of its ligands. To determine whether transgenic T cells that developed while overexpressing CTLA-4 were rendered inherently hyporesponsive or are inhibited in a ligand-independent manner, proliferation assays were performed using CHO cells that express I-Ek, but not B7.1 or B7.2. We hypothesized that CTLA-4 overexpression would not inhibit responses to these APCs, when the ligands for CD28 and CTLA-4 are not present. We found that CTLA-4 transgenic T cells respond comparably with littermate controls under these circumstances (Fig. 4C). These results demonstrate that CTLA-4 transgenic T cells are not inherently hyporesponsive, and imply that the lower responses seen are dependent on B7 expression. To confirm that the restricted response of CTLA-4 transgenic T cells is dependent on B7, we tested the response of CTLA-4 transgenic T cells when the interaction between B7 and its ligands was blocked. 5C.C7 and CTLA-4 transgenic 5C.C7 T cells were stimulated with L cells expressing I-Ek and B7.1 with or without CTLA-4 Ig to block B7 binding. We found that, when B7 was blocked, CTLA-4 transgenic and wild-type T cells responded equally to stimulation (Fig. 4D).

Multiple explanations exist for the decreased responsiveness of CTLA-4 transgenic T cells. First, CTLA-4 overexpression may effectively interfere with CD28-B7 interaction on the cell surface, thereby inhibiting T cell activation. Alternatively, increased CTLA-4 expression could increase the amount of negative signaling through CTLA-4 on these cells. To differentiate between these possibilities, we compared 5C.C7 rag2−/−/CD28−/− CTLA-4 transgenic and 5C.C7 rag2−/−/CD28−/− T cells. We used L cells expressing I-Ek and B7.1 as APCs and compared the T cell responses to MCC. The CTLA-4 transgenic cells responded equivalently to littermate controls in these assays (Fig. 4E), suggesting that the decreased responsiveness of these cells is CD28 dependent. One explanation is that CTLA-4 overexpression is outcompeting CD28 for B7 on account of its higher avidity. Because previous reports have shown that CTLA-4 can inhibit T cell responses in the absence of CD28 (36, 37), we speculate that a low level of CTLA-4 inhibition is occurring in both transgenic and littermate control T cells, and that increasing the amount of CTLA-4 on the T cells is not actually increasing the negative signal given by CTLA-4.

**CTLA-4 overexpression inhibits in vivo responses**

We next addressed the ability of the CTLA-4 transgene to inhibit in vivo responses. EAE is an autoimmune inflammatory disease that shares many clinical features with the human disease multiple sclerosis. Immunization of B6 mice with MOG35–55 leads to a CD4+ T cell-mediated inflammatory response in the CNS. As we have shown previously, CTLA-4 overexpression inhibits responses of CD4+ T cells. This would imply that CTLA-4 transgenic mice should be less susceptible to EAE than wild-type mice. To test this hypothesis, B6 CTLA-4 transgenic mice were compared with their littermate counterparts after immunization with MOG peptide. CTLA-4 transgenic mice exhibited increased survival and decreased severity of disease (Fig. 5 and Table II). This reduction of disease was despite the fact that CTLA-4 transgenics have fewer regulatory T cells than wild-type mice, suggesting that the effect of CTLA-4 on the activation of effector T cells was more important than the number of regulatory cells present in reducing autoimmunity.

**Discussion**

Despite the importance of CTLA-4 as a negative regulator of T cell activation, its mechanism of action is still unclear. At least five possibilities exist for how CTLA-4 inhibits T cell activity, three cell intrinsic mechanisms and two extrinsic. First, CTLA-4 may act as a B7 sink, effectively preventing B7-CD28 interactions because of its higher avidity. A second possibility is that CTLA-4 delivers a negative signal through its cytoplasmic tail that inhibits either TCR signaling, CD28 signaling, or both. Third is the idea of inhibitory lattice formation, where by virtue of its bivalent ligand binding, CTLA-4 may form a periodic structure that could interfere with normal synapse formation between the T cell and APC (38–40). Fourth, CTLA-4 may also have an important function in the suppressive effects of CD25+ regulatory T cells, thereby extrinsically restricting T cell function. Regulatory T cells have been shown to constitutively express surface CTLA-4, and experiments using bone marrow chimeric mice have shown that wild-type T cells can function to protect from the lymphoproliferation caused by CTLA-4-deficient T cells (41, 42). Fifth, CTLA-4 may function extrinsically by ligating B7 on APCs during Ag recognition, thereby up-regulating indoleamine 2,3-dioxygenase (IDO) (43), which is thought to inhibit T cell responses by depleting local tryptophan levels (44, 45). These mechanisms are not mutually exclusive, and it is likely that CTLA-4 functions in varying capacities at different times during an immune response.

**Table I. Summary of flow cytometry data**

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<th>Wild-Type B6</th>
<th>CTLA-4 Transgenic</th>
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<tr>
<td>% CD69&lt;sup&gt;+&lt;/sup&gt;</td>
<td>18.44 ± 1.67</td>
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</tr>
<tr>
<td>% CD44&lt;sup&gt;high&lt;/sup&gt;</td>
<td>15.83 ± 1.14</td>
<td>8.16 ± 1.26</td>
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<tr>
<td>% CD62L&lt;sup&gt;low&lt;/sup&gt;</td>
<td>12.63 ± 1.02</td>
<td>6.47 ± 0.99</td>
</tr>
<tr>
<td>% CD25&lt;sup&gt;+&lt;/sup&gt;</td>
<td>15.29 ± 0.83</td>
<td>3.94 ± 0.71</td>
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Transgenic overexpression of CTLA-4 is one method for evaluating how CTLA-4 functions as an inhibitor of T cell activation. In this study, we investigated the effects of CTLA-4 overexpression on T cells both in vivo and in vitro. CTLA-4 transgenic mice were generated and shown to detectably express CTLA-4 both intracellularly as well as on the surface of naive T cells. This is in contrast to wild-type T cells, which do not detectably express CTLA-4 either on the surface or intracellularly. These differences allow for the effect of CTLA-4 on naive T cells to be studied more effectively.

Our results demonstrate that transgenic overexpression of CTLA-4 has subtle effects on the population of T cells that developed in these mice. CTLA-4 is not normally expressed in the thymus of wild-type mice, but it is found in the cytoplasm of developing thymocytes in the CTLA-4 transgenic. Although CTLA-4 is not detected on the surface of CD4⁺CD8⁻ or CD4⁺CD8⁺ thymocytes in CTLA-4 transgenic mice, it is present in the cytoplasm. The trafficking pattern of CTLA-4 would suggest that it is expressed transiently at the surface before being internalized and should be able to interact with its ligands during this time (13–16).
FIGURE 4. CTLA-4 overexpression inhibits Ag-specific responses. A, 5C.C7 wild-type and 5C.C7 CTLA-4 transgenic T cells were mixed with mitomycin C-arrested L cells that express I-Ek and B7.1. A total of 50,000 T cells was mixed with 25,000 L cells and the indicated amount of MCC peptide. Cells were pulsed with [3H]thymidine at 72 h, and harvested 16 h later. B, 5C.C7 wild-type and 5C.C7 CTLA-4 transgenic T cells were mixed with irradiated splenocytes from B10.A mice. A total of 50,000 T cells was mixed with 100,000 splenocytes and the indicated amount of peptide. Cells were pulsed and harvested as above. C, 5C.C7 and 5C.C7 CTLA-4 transgenic T cells were mixed with mitomycin C-arrested CHO cells that express I-Ek. A total of 50,000 T cells was mixed with 25,000 CHO cells and the indicated amount of peptide. Cells were pulsed (Figure legend continues)
This expression correlates with an up-regulation of CD28 expression on CD4 single-positive thymocytes and mature lymph node T cells. The up-regulation of CD28 displayed in CD4 transgenic T cells is likely a compensation mechanism to allow for CD28 signaling in the presence of ectopic CTLA-4 expression. The role of CD28 in the development of T cells is controversial, with some data suggesting that CD28 signaling leads to negative selection of thymocytes (46), whereas other data suggest that CD28 shifts the dose response of TCR signaling in the thymus (47). The fact that CD28 is up-regulated on surviving CD4 single-positive T cells is inconsistent with a role for CD28 signaling in negative selection and suggests a positive role for CD28 in the development of CD4+ T cells.

Another interesting effect of CTLA-4 overexpression is a decrease in the number of CD25+ regulatory T cells compared with wild-type mice. This decrease is similar to what has been reported for CD28−/− mice, which have impaired development of CD25+ cells. Taken together with data demonstrating that CD28 signaling is required for Foxp3 expression, a transcription factor necessary for the development of CD25+ regulatory cells, our results suggest that CTLA-4 transgenic mice have decreased numbers of regulatory cells because CTLA-4 is inhibiting the CD28-B7 interactions necessary for their development. Although it cannot be ruled out that CTLA-4 signaling directly inhibits the development of these cells in a CD28-independent manner, this seems unlikely because CTLA-4 is normally expressed on these cells.

In all, our results suggest that the inhibition of immune responses in CTLA-4 transgenic mice seemed to be due to prevention of B7-CD28 interaction and/or CD28 signaling. Increased expression of CTLA-4 did not inhibit T cell responses when CD28 was not present on T cells or when B7 was not present on APCs. If APCs were used that did not express B7 or if B7-CD28/CTLA-4 interactions were blocked, then transgenic T cells were equally responsive to wild-type T cells. When CD28−/− T cells that also overexpressed CTLA-4 were compared with CD28−/− control T cells, we found that transgenic cells were not inhibited by CTLA-4 overexpression. This finding appears to be inconsistent with the previous finding that CTLA-4 can inhibit T cell responses in the absence of CD28 (36, 37). There are several possible explanations for this discrepancy. First, there may be a low level of inhibition by endogenous CTLA-4 that is occurring in both the CD28 transgenic and wild-type mice, and increasing the amount of CTLA-4 on the surface does not increase this negative signal. Another possibility is that, in the absence of CD28, T cells do not become sufficiently activated for CTLA-4 to have an effect on cell cycle progression. Also, surface CTLA-4 expression may not necessarily correlate with CTLA-4 expression at the synapse, and it is possible that CTLA-4 may need to be in the synapse to inhibit intracellular signaling events. Our data suggest that CTLA-4 overexpression does not inhibit T cell responses in the absence of CD28, because overexpressed CTLA-4 primarily functions by outcompeting CD28 for ligand. None of these possibilities conflicts with the notion that endogenous CTLA-4 can inhibit T cell responses in the absence of CD28. We cannot rule out that CTLA-4 only inhibits a CD28-specific signaling pathway; however, current data do not support the existence of this pathway (48, 49). Given that CTLA-4 has a higher affinity for B7 than CD28, the ligand competition scenario seems most plausible. Thus, we believe these findings reveal a primary mechanism of inhibition by a CTLA-4 transgene and confirm previous results that CTLA-4 can function as an inhibitor based on its ability to outcompete CD28 for B7 ligation.

A recent study has addressed the role of CTLA-4 expression in the development of autoimmune disease in IL-2−/− mice (50). In this study CTLA-4 transgenic overexpression was shown to partially rescue the lymphoproliferative disorder seen in IL-2−/− mice. It was postulated that this rescue was the result of restoring CTLA-4 expression, which is normally not up-regulated in IL-2−/− mice. However, the ability of CTLA-4 overexpression to rescue the lymphoproliferation in IL-2−/− animals is not surprising, because increased CTLA-4 expression will inhibit T cell responses regardless of IL-2 deficiency. Indeed, overexpression of another inhibitory receptor, Ly49A, can partially rescue the lymphoproliferative phenotype of CTLA-4-deficient animals, demonstrating that unrelated pathways can compensate for each other (51). In addition to demonstrating that CTLA-4 overexpression can compensate for IL-2 deficiency, these studies also found that CD28−/− CTLA-4 transgenic T cells were less responsive than CD28−/− wild-type cells. This finding is in contrast to our studies

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**Table II. Summary of EAE results**

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<th>Wild-Type B6</th>
<th>CTLA-4 Transgenic</th>
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<tr>
<td>Number of mice</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Survival*</td>
<td>13/18</td>
<td>18/18</td>
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<tr>
<td>Incidence</td>
<td>18/18</td>
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<tr>
<td>Mean peak clinical score**</td>
<td>3.9</td>
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*, P < 0.05; **, P < 0.0001.
where inhibition by the CTLA-4 transgene was dependent on CD28 expression. One reason for the discrepancy could be that, in the previous study, syngeneic APCs and anti-CD3 Ab were used to stimulate T cells. This allows for the possibility that CTLA-4 on the surface of the transgenic cells could have an effect on the APC. Recent data have shown that B7 ligation by CTLA-4 can up-regulate IDO expression on dendritic cells (43), which is thought to inhibit T cell responses by depletion of local levels of tryptophan (44). In our system, using L cells that have not been shown to up-regulate IDO, any effects by CTLA-4 would likely be T cell intrinsic. Our data would suggest that overexpression of CTLA-4 is inhibiting the CD28-B7 interactions critical for the activation of naive T cells. This could also explain the decrease in autoreactivity seen in IL-2−/−, CTLA-4 transgenic mice.

CTLA-4 is expressed and can function to inhibit the T cell response at very early time points following TCR engagement. Although transgenic overexpression of CTLA-4 on naive T cells may be nonphysiological, it does provide the ability to investigate the mechanisms of CTLA-4 inhibition during the initial stages of T cell activation. We show here that overexpression of CTLA-4 on naive T cells inhibits T cell responses both in vitro and in vivo through a mechanism that we believe primarily involves the ability of CTLA-4 to outcompete CD28 for ligand binding and is independent of which B7 is the predominant ligand available. Thus, prevention of CD28-mediated costimulation by acting as a B7 sink may function as an important mechanism for CTLA-4-mediated inhibition during the initial activation of a naive T cell.

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References


