Activated T cells express inhibitory receptors such as CTLA-4 that can downregulate immune responses. Blockade of or genetic deficiency in CTLA-4 can result in autoimmunity. Therefore, strategies to increase the inhibitory function of CTLA-4 may be attractive in settings of undesirable T cell responses such as autoimmunity or transplant rejection. We have tested the hypothesis that transgenic constitutive expression of CTLA-4 can further attenuate immune responses when compared with normal inducible expression. Our results indicate that transgenic expression of CTLA-4 in mouse T cells (CTLA-4-Tg T cells) results in reduced cell cycle progression and increased apoptosis of TCR-stimulated T cells. CTLA-4-Tg T cells display reduced T cell proliferation in an in vivo model of graft versus host disease (GVHD). These results further our understanding of how CTLA-4 can be manipulated to inhibit immune responses and may help development of new therapeutic strategies for clinical settings of autoimmunity and transplantation.

Key Words: Cell surface molecules, Cellular activation, T lymphocytes, Transgenic, Rodent

INTRODUCTION

Tightly controlling T cell activation is crucial to the prevention of autoimmune diseases and lymphoproliferative syndromes. T cells become activated following recognition by the TCR of antigenic peptides presented by MHC molecules on APCs, concurrently to the ligation of costimulatory receptors. CD28, the prototype of T cell costimulatory receptors, is constitutively expressed on T cells and binds B7-1 (CD80) and B7-2 (CD86) on APCs [1]. Once activated, T cells upregulate several surface receptors that, when engaged, limit immune responses by inducing either cell-cycle arrest or apoptosis. This is thought to be essential to prevent uncontrolled progressive lymphocaccumulation. CTLA-4 is one such inhibitory receptor that is transiently expressed after T cell activation. Like CD28, CTLA-4 binds B7-1 and B7-2, albeit with a higher affinity than CD28 [1]. Thus, it is thought that endogenous CTLA-4 can inhibit T cell responses both by preventing CD28 from binding its ligands as well as by directly inhibiting TCR signaling [2,3].

Consistent with the inhibitory role of CTLA-4, CTLA-4-deficiency in mice results in a severe lymphoproliferative disorder and an autoimmune disease that leads to early animal death [4,5]. Similarly, blockade of CTLA-4 engagement following administration of anti-CTLA-4 mAbs in vivo leads to increased severity of disease in mouse models of autoimmunity, such as diabetes [6] and experimental autoimmune encephalopathy [7]. Thus, the converse approach of augmenting CTLA-4 signaling would be desirable to inhibit immune responses in settings of autoimmune diseases or of transplant rejection. However, few reagents to date can cross-link CTLA-4 in vivo and elicit its negative regulatory effect on T cell function. We have previously shown that expression of a membrane-bound single chain anti-CTLA-4 mAb on a tumor cell-line effectively engaged CTLA-4 and led to reduced allogeneic tumor rejection when these tumor cells were inoculated into MHC-mismatched recipients [8]. These results indicate that endogenous CTLA-4 can be cross-linked more effectively than what normally occurs during antigen encounter in vivo, resulting in significantly dampened immune responses.

In this study, we explored the possibility that increasing the expression levels of CTLA-4 and having its expression be constitutive rather than induced in T cells may also result in further inhibition of T cell responses. CTLA-4 transgenic (CTLA-4-Tg) mice that constitutively express a CTLA-4 transgene in addition to the inducible endogenous CTLA-4 were previously generated [9]. When crossed with CTLA-4-
METHODS

Mice

CTLA-4-Tg mice (H-2d) were crossed with CD28-deficient (CD28-KO) (H-2d) mice (Jackson Laboratories, Bar Harbor, ME). Control littersmates were used in all experiments and termed wildtype (WT), CTLA-4-Tg, CD28-KO or CTLA-4-Tg/CD28-KO. BALB/c mice (H-2d) were obtained from Frederick Cancer Research Center (Frederick, MD). Animals were kept under specific pathogen-free conditions and utilized in agreement with the Institutional Animal Care and Use Committee, according to the NIH guidelines for animal use.

Proliferation assays

Splenocytes (2×10^5/well) from WT and CTLA-4-Tg mice or from CD28-KO and CTLA-4-Tg/CD28-KO mice were incubated with various concentrations of anti-CD3 mAb. Plates were pulsed with ^3H-thymidine (1 μCi/well) for the last 6~0 h of 72 h culture, as indicated in the text. ^3H-thymidine incorporation was measured using a TopCount™ NXTTM microplate scintillation and luminescence counter with TopCount™ NXTTM software (Packard BioScience, Meriden, CT).

Flow cytometry analysis

To determine the intracellular DNA content, CD4^-^ and CD8^-^ WT and CTLA-4-Tg T cells were enriched from spleen by negative selection over a magnetic column according to the instructions of the manufacturer (StemCell, Vancouver, Canada). T cells (5×10^7/well) were stimulated with anti-CD3 mAb (1 μg/ml) in the presence of T-depleted (anti-Thy 1.2^-^ rabbit complement), irradiated (2,000 rads) WT splenocytes (2.5×10^6/well). T cells were harvested at different time points and stained with FITC-coupled anti-CD4 or anti-CD8 mAbs (BD/PharMingen, San Diego, CA). The data were analyzed using Cell Quest and Flow Joe softwares (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

For cell proliferation analysis, CD28-KO and CTLA-4-Tg/CD28-KO T cells enriched by negative selection were labeled with CFSE. Briefly, 5×10^5 cells were washed once and resuspended in PBS at a concentration of 2×10^5/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 9 minutes. The reaction was quenched by the addition of 5 ml of FCS for 1 minute. Cells were then washed twice in complete medium and stimulated as above. Cells were harvested at different time points, stained with PE coupled anti-CD4 or anti-CD8 mAbs (BD/PharMingen).

GVHD model

BALB/c mice were sub-lethally irradiated (650 rads). After 24 h, the animals received an intravenous injection of CD28-KO or CTLA-4-Tg/CD28-KO T cells (4~6×10^6 cells/mouse) that had been purified by negative selection and labeled with CFSE as described above. The animals were sacrificed on day 2 and spleens were harvested. Splenocytes labeled with PE-coupled anti-K^b^ and APC-coupled anti-Thy1.2, CD4 or CD8 (BD/PharMingen) and analyzed by flow cytometry. Events were gated on K^b^/Thy1.2^-^, K^b^/CD4^-^, or K^b^/CD8^-^ cells and CFSE fluorescence intensity was measured.

RESULTS

Overexpression of CTLA-4 in T cells results in reduced responses of T cells in vitro

To determine whether constitutive overexpression of CTLA-4 in T cells resulted in reduced T cell responses, splenocytes from WT and CTLA-4-Tg mice were utilized. Our previous experiments have demonstrated that CTLA-4-Tg CD4^-^ and CD8^-^ T cells constitutively express high levels of CTLA-4, that are comparable to those on 72 h activated WT T cells. T cell activation of CTLA-4-Tg T cells further increases this

Fig. 1. Reduced proliferation by CTLA-4-Tg splenocytes. Splenocytes from WT and CTLA-4-Tg mice were incubated with soluble anti-CD3 mAbs and incorporation of ^3H-thymidine for the last 6 h of a 72 h culture was assessed. The data are expressed as the means±SD. The result is representative of 3 independent experiments.
Expression of CTLA-4 Reduces T Cell Responses

Fig. 2. TCR-stimulated CTLA-4-Tg T cells have reduced cell-cycle progression and increased apoptosis than WT T cells. CD4⁺ T cells were purified from WT and CTLA-4-Tg splenocytes and stimulated with anti-CD3 (1 μg/ml) in the presence of T-depleted irradiated syngeneic WT splenocytes. Cells were stained with anti-CD4-APC, fixed, permeabilized, stained with PI and analyzed by flow cytometry. Doubles were excluded based on FL2-Width. The histograms represent PI fluorescence intensity on CD4⁺-gated cells. The numbers in the upper right corner of the plot represent the percent of CD4⁺ cells in S/G2/M phases of the cell-cycle. The numbers in the upper left corner represent the percent of CD4⁺ cells with subdiploid DNA content. This result is representative of 3 independent experiments.

Fig. 3. Greater number of CTLA-4-Tg than WT T cells undergoes apoptosis following TCR stimulation. T cells were purified from WT and CTLA-4-Tg splenocytes and stimulated as for Fig. 2. Cells were stained with APC-conjugated antiCD4 or anti-CD8, FITC-coupled annexin V and PI and analyzed by flow cytometry. Dot plots represent annexin V versus PI fluorescence of cells following gating on CD4⁺ or CD8⁺ cells. Numbers in the plots represent percent CD4⁺ or CD8⁺ cells that stain positive for annexin V (lower right quadrant) or for annexin V and PI (upper right quadrant). This result is representative of 2 independent experiments.

level of expression (data not shown). As shown in Fig. 1, anti-CD3 mAb-stimulated CTLA-4-Tg cells displayed reduced ³H-thymidine incorporation than WT cells, suggesting that overexpression of CTLA-4 can suppress mitogen-induced T cell responses in vitro.

**CTLA-4-Tg T cells display reduced cell-cycle progression and increased death upon TCR stimulation**

Decreased anti-CD3-dependent ³H-thymidine incorporation could be the consequence of reduced T cell-cycle progression or of augmented T cell death. In addition, it was possible that APCs from CTLA-4-Tg mice had reduced antigen presentation or costimulatory capacity because of chronic exposure to CTLA-4. Therefore, to assess the capacity of T cells to progress through the cell-cycle, CD4⁺ T cells were purified from WT and CTLA-4-Tg splenocytes and stimulated with anti-CD3 mAb. To normalize the APCs in the system, irradiated T-depleted syngeneic WT splenocytes (syngeneic APCs) were used. T cells were then subjected to intracellular PI staining at different time points after stimulation. As shown in Fig. 2, fewer CD4⁺ CTLA-4-Tg T cells were in the S-G2-M phases of the cell cycle both at 48 and 72 h after activation when compared with WT T cells. In addition, a greater percentage of CTLA-4-Tg than WT T cells were subdiploid and thus likely apoptotic at 72 h. Similar results were obtained at 72 h when using CD8⁺ T cells (data not shown).

To confirm that expression of the CTLA-4 transgene resulted in increased T cell death, purified T cells were stimu-
Our study shows that enhancing the level of expression of CTLA-4 would also inhibit antigen-mediated immune responses in vivo. To address this question, a GVHD model was developed. Recipient BALB/c mice (H-2d) were sub-lethally irradiated and adoptively transferred with CFSE-labeled purified T cells from CD28-KO or CTLA-4-Tg/CD28 KO mice (H-2d). Animals were sacrificed on day 2 and division of transferred T cells was analyzed by flow cytometry. Reduced expansion of CTLA-4-Tg/CD28-KO T cells than CD28-KO T cells was observed both in CD4+ and CD8+ T cells (Fig. 4). This result indicates that overexpression of CTLA-4 can downregulate T cell responses independent of CD28 expression in vivo.

DISCUSSION

Our study shows that enhancing the level of expression of CTLA-4 in T cells can result in reduced T cell responses both in vitro and in vivo. CTLA-4 has been widely used as an immunological target in experimental tumor models since its blockade, following systemic administration of an anti-CTLA-4 mAb, was reported to augment immune responses [11]. Two clinical trials using this approach were recently released and showed promising results as preventing CTLA-4 engagement in tumor patients was demonstrated to augment T cell responses, and, in some cases, lead to partial or complete tumor regression [12,13]. In contrast, finding means to mimic the inhibitory function of CTLA-4 in vivo, as a therapeutic method to attenuate or prevent autoimmune flairs or transplant rejection episodes, has proven elusive. The approaches that we and others have published to date are based on transforming blocking anti-CTLA-4 mAbs into agonistic mAbs by use of bi-specificity [14,15] or plasma membrane immobilization [8], or by membrane-immobilizing a mutated form of B7-1 that binds CTLA-4 but not CD28 [16]. In this study, we have examined whether forcing constitutive high levels of CTLA-4 expression in T cells is sufficient to downregulate immune responses. Previous work from Pecotte and colleagues has shown that upregulation of endogenous CTLA-4 may be one of the mechanisms responsible for tolerance after administration of anti-CD45RB mAb in a mouse transplant model [17], suggesting that enhancing CTLA-4 expression in T cells may be a valid approach to inhibit T cell responses. Our current work supports these conclusions and indicates that genetically forcing expression of CTLA-4 such that it is expressed constitutively in all T cells results in further inhibition of T cell immune responses.

Whereas it is clear that engagement of endogenous CTLA-4 can induce cell-cycle arrest by inhibiting cyclin D3, CDK4 and CDK6 [18], whether CTLA-4 ligation induces T cell death is more controversial. Only two reports to date have supported apoptosis of human or mouse T cells after engagement of endogenous CTLA-4 in activated T cells [19,20]. In contrast, engagement of CTLA-4 was reported to block CD95-mediated cell death [21] and to maintain T cell survival by allowing TCR-mediated expression of Bcl-xL [22]. Our results suggest that constitutive expression of CTLA-4 can result in increased T cell death. Whether this is due to growth factor deprivation as engagement of CTLA-4 results in reduced cytokine production by T cells, or to increased susceptibility to apoptosis downstream of death receptors remains to be investigated.

It has been previously shown that endogenous CTLA-4 can function in a CD28 independent manner, as blockade of CTLA-4 in vivo results in accelerated heart allograft rejection in CD28-deficient mice [2]. Also, it has been reported that CTLA-4 could inhibit T cell function in the absence of CD28 [23]. There has been a contrasting report however, that CTLA-4 overexpression is unable to attenuate immune responses in the absence of CD28 [24]. We have previously shown that T cells in CTLA-4-Tg/CD28 KO mice exhibit lower proliferation levels compared to their counterparts in CD28-KO mice without regard to IL-2 concentrations [25]. Our results in this current study indicates that constitutive transgenic expression of CTLA-4 can also inhibit T cell responses independently of CD28 expression in vivo, even though its constitutive expression would be expected to efficiently prevent CD28 ligation to B7. One possibility is that B7 scavenging from CD28 is not very efficient in naive T cells because the cellular distribution of the CTLA-4 transgene is similar to that of endogenous CTLA-4 after T cell activation. Most of the CTLA-4 molecules are found in intracellular compartments and very few molecules are displayed on the T cell surface at any given point (data not shown). As the CTLA-4 transgene comprises the same cytoplasmic tail as the endogenous protein, it is likely that it will follow the same rules. The motif surrounding tyrosine...
201 enables CTLA-4 to bind to the clathrin adaptor AP-50, resulting in rapid endocytosis from the cell surface [26,27]. This process is prevented transiently when tyrosine 201 is phosphorylated following TCR stimulation, leading to increased CTLA-4 surface accumulation [28]. Therefore, it is likely that the CTLA-4 transgene on naive T cells may not bind B7 molecules very effectively despite its high affinity for B7 family members.

We have recently shown that the CTLA-4 transgene can prevent lymphoproliferation and autoimmunity in IL-2-deficient mice, an autoimmune model with reduced CTLA-4 expression in T cells [25]. These results had suggested that augmenting CTLA-4 expression in T cells could have clinical application potential in autoimmune situations of deficient CTLA-4 upregulation. Our current study suggests that augmenting CTLA-4 expression in T cells may be an attractive strategy to reduce T cell responses in autoimmunity or transplantation settings, even if endogenous expression of CTLA-4 is normal.

ACKNOWLEDGEMENTS

This research was supported by the Chung-Ang University Research Grants in 2009.

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