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CTLA-4-Tg/CD-28-KO Mice Exhibit Reduced T Cell Proliferation *in vivo* Compared to CD-28-KO Mice in a Graft-versus-host Disease Model

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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 lymphoaccumulation. 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-

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ABBREVIATIONS: APC, antigen presenting cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; FITC, fluorescein isothiocyanate; CTLA-4, cytotoxic T-lymphocyte antigen 4; MHC, major histocompatibility complex; TCR, T cell receptor.

deficient mice, these transgenic animals were free of detectable lymphoproliferation and autoimmunity, indicating that expression of the transgene could replace that of endogenous CTLA-4. Thus, although transgenic expression of CTLA-4 in T cells reduced the severity of spontaneously occurring autoimmune diseases, it was not clear whether it would also reduce T cell responses after exogenous antigenic challenge. In addition, the autoimmune disease models could not distinguish whether the inhibitory effects of constitutive CTLA-4 overexpression were due to B7 scavenging away from CD28, or to intrinsic inhibitory properties of the transgene. Indeed, treatment of CTLA-4-deficient mice from birth with soluble CTLA-4-Ig that blocked CD28 ligation is sufficient to prevent disease [10]. In this study, we investigated whether constitutive expression of CTLA-4 can inhibit T cell activation in settings of CD28-sufficiency and -deficiency *in vivo*.

METHODS

Mice

CTLA-4-Tg mice (H-2^b) were crossed with CD28-deficient (CD28-KO) (H-2^b) mice (Jackson Laboratories, Bar Harbor, ME). Control littermates were used in all experiments and termed wildtype (WT), CTLA-4-Tg, CD28-KO or CTLA-4-Tg/CD28-KO. BALB/c mice (H-2^d) 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 ^{3}H-thymidine (1 μCi/well) for the last 6~8 h of 72 h culture, as indicated in the text. ^{3}H-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 (5×10^4/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 (2.5×10^5/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). Cells were then incubated in paraformaldehyde (10% in PBS) for 10 minutes at room temperature, washed and incubated in ice-cold methanol for 20 minutes at −20°C. The cells were washed twice, resuspended in RNAse (l mg/ml in PBS) and incubated at 37°C for 30 minutes. Cells were then washed and resuspended in propidium iodide (PI, 2.5 μg/ml, Sigma-Aldrich, Milwaukee, WI) and immediately analyzed by flow cytometry. 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, 5×10^6 cells were washed once and resuspended in PBS at a concentration of 2×10^7/ml. An equal volume of a CFSE (Molecular Probes, Eugene, OR) solution (5 μM in PBS) was added and cells were incubated at room temperature for 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 ($4\sim6\times10^6$ 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 were 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

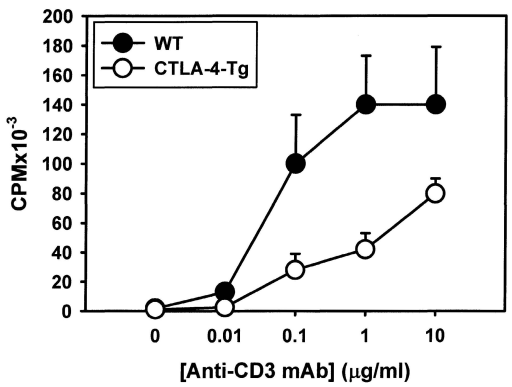

Fig. 1. Reduced proliferation by CTLA-4-Tg splenocytes. Splenocytes from WT and CTLA-4-Tg mice were incubated with soluble anti-CD3 mAb and incorporation of ^{3}H-thymidine for the last 6 h of a 72 h culture was assessed. The data are expressed as the mean± SD. The result is representative of 3 independent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

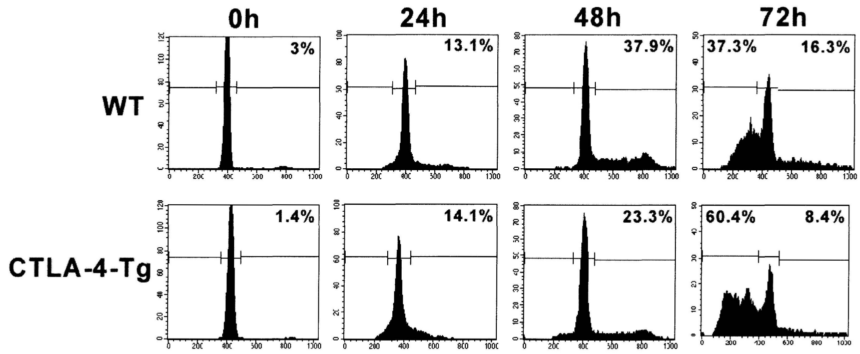

Fig. 2. TCR-stimulated CTLA-4-Tg T cells have reduced cell-cycle progression and increased apoptosis than WT T cells. $CD4^+$ T cells were purified from WT and CTLA-4-Tg splenocytes and stimulated with anti-CD3 (1 μg/ml) in the presence of T-depleted irradiated syngeneic WT splenocytes. Cells were stained with anti-CD4-APC, fixed, permeabilized, stained with PI and analyzed by flow cytometry. Doublets 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.

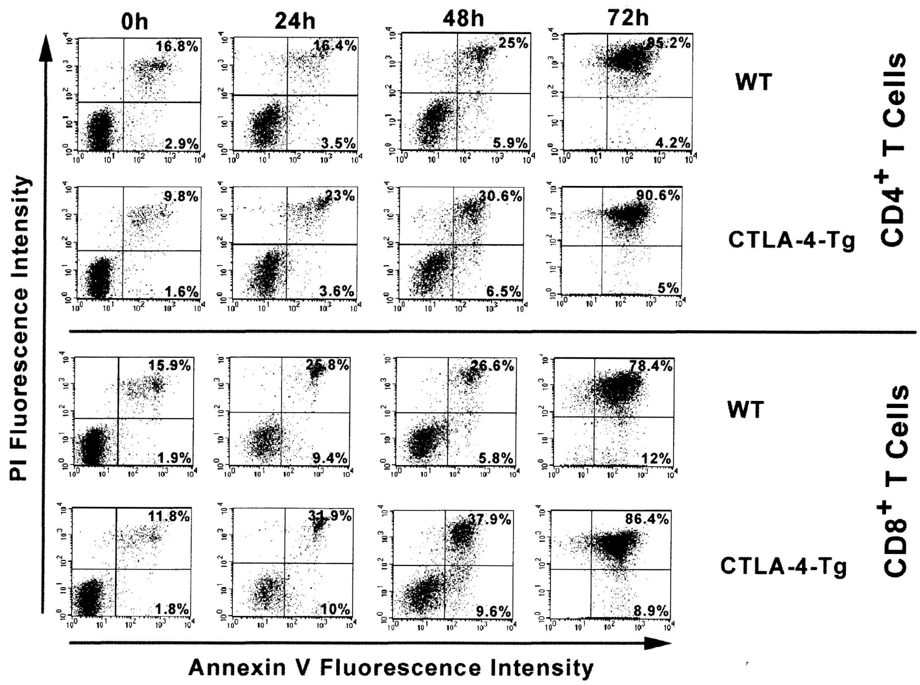

Fig. 3. Greater number of CTLA-4-Tg than WT T cells undergoes apoptosis following TCR stimulation. T cells were purified from WT and CTLA-4-Tg splenocytes and stimulated as for Fig. 2. Cells were stained with APC-conjugated antiCD4 or anti-CD8, FITC-coupled annexin V and PI and analyzed by flow cytometry. 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 ^{3}H-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 ^{3}H-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-

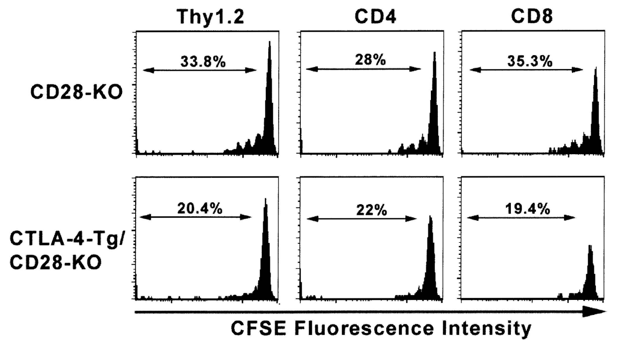

Fig. 4. Reduced *in vivo* responses by CTLA-4-Tg/CD28-KO than CD28-KO T cells in a GVHD model. T cells were purified from CD28-KO and CTLA-4-Tg/CD28 KO splenocytes and labeled with CFSE. 4×10^6 T cells were injected intravenously into sub-lethally irradiated BALB/c recipients. Animals were sacrificed on day 2 and splenocytes were stained with PE-coupled anti-K^b and APC-coupled anti-Thy 1.2, CD4, or CD8 mAbs. Events were gated on K^{b+}/Thy 1.2^+, $K^{b+}/CD4^+$, or $K^{b+}/CD8^+$ cells and CFSE fluorescence are displayed as histograms. Results are representative of 2 independent experiments.

lated with anti-CD3 mAb in the presence of syngeneic APCs. Cells were harvested at different time points, stained with annexin V-FITC and PI and analyzed by flow cytometry. As suggested by the intracellular PI staining results, a greater percentage of both $CD4^+$ and $CD8^+$ T cells were found to be positive for annexin V and PI staining at all time points after activation (Fig. 3). These results indicate that constitutive overexpression of CTLA-4 in T cells promotes both decreased cell-cycle progression and increased cell death following TCR stimulation *in vitro*.

CTLA-4-Tg T cells have reduced T cell responses in vivo in the absence of CD28 expression

Finally, we wanted to determine if constitutive overexpression 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 ($H\text{-}2^d$) were sub-lethally irradiated and adoptively transferred with CFSE-labeled purified T cells from CD28-KO or CTLA-4-Tg/CD28-KO mice ($H\text{-}2^b$). 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 Fecteau 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 inhibitin 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 $Bcl\text{-}x_L$ [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.

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