

Transgenic Expression of CTLA-4 Controls Lymphoproliferation in IL-2-Deficient Mice¹

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IL-2-deficient mice develop a lymphoproliferative and autoimmune disease characterized by autoimmune hemolytic anemia (AHA) and inflammatory bowel disease. We have previously reported that IL-2 is necessary for optimal up-regulation of CTLA-4, an inducible negative regulator of T cell activation. In this study, we have tested the hypothesis that reduced expression of CTLA-4 in IL-2-deficient T cells contributes to the pathogenesis of disease in IL-2-deficient mice. Expression of *CTLA-4* as a transgene completely prevented lymphoaccumulation and AHA in IL-2-deficient mice. The normalization of T cell numbers was due to inhibition of expansion of conventional CD4⁺CD25⁻ T cells rather than to rescue of the numbers or function of CD4⁺CD25⁺ regulatory T cells, suggesting that CTLA-4 expression on conventional T cells plays a role in maintaining normal T cell homeostasis. In addition, the inhibitory effect of the *CTLA-4* transgene on T cell expansion was at least in part independent of CD28 expression. Our results suggest that deficient CTLA-4 expression on conventional T cells contributes to the pathophysiology of the lymphoproliferative disease and AHA in IL-2-deficient mice. Thus, restoring CTLA-4 expression in T cells may be an attractive strategy to control clinical autoimmune diseases in which CTLA-4 expression is reduced. *The Journal of Immunology*, 2004, 173: 5415–5424.

The activation and quiescence of T lymphocytes is a tightly controlled phenomenon postulated to be important in maintaining T cell homeostasis. T cells are activated following engagement of their TCR by specific peptide/MHC and concurrent ligation of costimulatory receptors such as CD28 (1). Inappropriate expansion of activated T cells may be prevented by several mechanisms including the activation of death-promoting receptors such as Fas (CD95) (2); the up-regulation of inhibitory molecules such as CTLA-4 (1), programmed death-1 (3), and B and T lymphocyte attenuator (4), the engagement of which can trigger cell cycle arrest; and the presence of regulatory cells that can suppress the expansion of conventional T cells (5). This control is important to prevent progressive lymphoaccumulation, because mice deficient in Fas (6), CTLA-4 (7, 8), programmed death-1 (9, 10), and B and T lymphocyte attenuator (4) expression develop different degrees of lymphoaccumulation and autoimmunity. Similarly, mice deficient in a subset of regulatory cells (CD4⁺CD25⁺ regulatory T cells (Treg)³) because of a genetic mutation in *FoxP3*, a tran-

scription factor thought to be necessary for generation of Treg, also develop massive lymphoproliferation (11).

CTLA-4 binds the same ligands as CD28, B7-1 (CD80), and B7-2 (CD86), but with higher affinity (1). In contrast to CD28, which is expressed in naive T cells, CTLA-4 is expressed only after T cell activation in conventional T cells and inhibits T cell responses both by blocking CD28 engagement with B7 ligands and by directly transducing negative signals to T cells (12). CTLA-4-deficient mice develop massive lymphoaccumulation in secondary lymphoid organs and T cell infiltration of different tissues leading to animal death before 4 wk of age (7, 8). We have previously shown that up-regulation of CTLA-4 following T cell activation depends on IL-2-mediated signals (13). Indeed, IL-2R blockade prevented CTLA-4 expression following activation of wild-type T cells, and IL-2-deficient T cells were markedly impaired in their ability to up-regulate CTLA-4. Interestingly, IL-2-deficient mice also develop a lymphoproliferative disease (14, 15). A similar disease is observed in mice deficient in IL-2R α (CD25) (16) and IL-2R β (17). The cause of the disease in IL-2 signaling-deficient mice is now thought to be multifactorial, and two causes are commonly cited (18). First, T cells deficient in IL-2 signaling have decreased susceptibility to Fas-mediated cell death (19). This may be due to reduced expression of Fas ligand after T cell activation and to absence of down-regulation of the putative inhibitor of Fas-induced apoptosis, c-FLIP (20). Second, IL-2 signaling-deficient mice have reduced numbers of Treg (21), and the transfer of Treg wild-type T cells has been shown to prevent Ag-induced hyperlymphoaccumulation of DO11.10/IL-2-deficient T cells (22). In addition, in the case of IL-2R β -deficient mice, disease is prevented by thymic expression of IL-2R β , which correlates with restoration of normal numbers of peripheral Treg (23, 24), and transfer of wild-type Treg into neonatal IL-2R β -deficient mice prevents disease onset.

Because IL-2-mediated signals are critical for CTLA-4 up-regulation on conventional T cells, we hypothesized that reduced expression of CTLA-4 in IL-2-deficient T cells may also participate

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³ Abbreviations used in this paper: Treg, CD4⁺CD25⁺ regulatory T cell; Tg, transgenic; KO, knockout; WT, wild type; PI, propidium iodide; AHA, acute hemolytic anemia; IBD, inflammatory bowel disease; RANK, receptor activator of NF- κ B; RANKL, ligand for RANK.

in the pathogenesis of the lymphoproliferation and autoimmunity in IL-2-deficient mice. To determine whether forced expression of CTLA-4 could prevent disease in IL-2-deficient animals, we generated CTLA-4 transgenic (Tg)/IL-2-deficient (knockout (KO)) (CTLA-4-Tg/IL-2-KO) mice in which the *CTLA-4* transgene is constitutively expressed in T cells under the control of the Lck proximal promoter and CD2 locus control region, and is not affected by presence or absence of IL-2. Our results indicate that forced expression of CTLA-4 in T cells prevents lymphoaccumulation in IL-2-deficient mice, and that these effects are likely due to a direct action of CTLA-4 on conventional peripheral T cells, rather than on Treg.

Materials and Methods

Mice

CTLA-4-Tg/CTLA-4-deficient mice (H-2^b) have been previously described (25) and were crossed with IL-2-deficient (H-2^b) or CD28-deficient (H-2^b) mice (purchased from The Jackson Laboratory (Bar Harbor, ME)). The CTLA-4 deficiency was eliminated from the genotype by further backcrossing. CTLA-4-Tg^{+/-}IL-2^{+/-} mice were maintained in breeding to produce wild-type (WT), CTLA-4-Tg, IL-2-KO, and CTLA-4-Tg/IL-2-KO littermates, and CTLA-4-Tg^{+/-}CD28-KO mice were maintained in breeding to produce CD28-KO and CTLA-4-Tg/CD28-KO mice. In all experiments described in this study, "WT mice" refers to CTLA-4-Tg^{negative}IL-2^{+/+} control littermates. RAG2-deficient (H-2^b) mice were maintained in our animal facility. Animals were kept under specific pathogen-free conditions and used in agreement with the Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Flow cytometry

To determine total CTLA-4 expression levels, spleens were harvested from 6- to 8-wk-old animals. Splenocytes were freshly analyzed or incubated with soluble anti-CD3 mAb (10 μ g/ml) for 72 h. Cells were fixed and maintained intact or permeabilized as previously described (26), and stained using FITC-coupled anti-CD4 or anti-CD8 and PE-coupled isotype control or anti-CTLA-4 mAb (BD Pharmingen, San Diego, CA). Two-color flow cytometry was performed using a FACSCalibur flow cytometer (BD Immunocytometry Systems, Mountain View, CA). Live cells were collected based on forward and side scatter. Data analysis was performed using CellQuest software (BD Biosciences, Mountain View, CA).

To determine counts of T cell subsets over time, single-cell suspensions taken from 7- and 16-wk-old mice were counted under the microscope using the trypan blue exclusion method, and the total number of live cells counted was multiplied by the percentage of CD4⁺ and CD8⁺ T cells as determined by flow cytometric analysis of nonpermeabilized live-gated cells.

To determine the intracellular DNA content, CD4⁺ and CD8⁺ T cells were enriched by negative selection over a magnetic column according to the instructions of the manufacturer (StemCell Technologies, Vancouver, BC, Canada). T cells were incubated in paraformaldehyde (10% in PBS) for 10 min at room temperature, washed, and incubated in ice-cold methanol for 20 min at -20°C. The cells were washed twice, resuspended in RNase (1 mg/ml in PBS), and incubated at 37°C for 30 min. Cells were then washed and resuspended in propidium iodide (PI; 2.5 μ g/ml) and immediately analyzed by flow cytometry. The data were analyzed using CellQuest and FlowJo softwares.

To determine the level of CTLA-4 expression in CD4⁺CD25⁺ T cells, splenocytes were stained with dialyzed anti-CD4-allophycocyanin and anti-CD25-FITC (BD Pharmingen), and CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were sorted using a fast speed cell sorter (MoFlo; BD Biosciences). Purity was typically 92–95%. Sorted populations were fixed, permeabilized, and stained with PE-coupled control IgG or anti-CTLA-4 mAb, as described above.

For cell proliferation analysis, CD28-KO and CTLA-4-Tg/CD28-KO T cells enriched by negative selection were labeled with CFSE (Molecular Probes, Eugene, OR). Briefly, 5 \times 10⁶ cells were washed once and resuspended in PBS at a concentration of 2 \times 10⁷/ml. An equal volume of a CFSE solution (5 μ M in PBS) was added, and cells were incubated at room temperature for 9 min. The reaction was quenched by the addition of 5 ml of FCS for 1 min. Cells were then washed twice in complete medium and stimulated as above. Cells were harvested at different time points, and stained with PE-coupled anti-CD4 or anti-CD8 mAbs (BD Pharmingen).

In vitro suppression assays

Splenic CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were isolated by high-speed cell sorting. WT CD4⁺CD25⁻ cells were used as responders (2 \times 10⁴/well) and were cultured in the presence of anti-CD3 mAb (10 μ g/ml) and irradiated T-depleted syngeneic APCs (1 \times 10⁵/well). A dose titration of CD4⁺CD25⁺ or CD4⁺CD25⁻ cells from the different genotypes was added in selected wells to serve as regulatory cells. Plates were pulsed with [³H]thymidine (1 μ Ci/well) for the last 6 h of a 72-h culture.

Adoptive transfer experiments

Splenic CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were isolated by high-speed cell sorting. CD4⁺CD25⁻ (0.4–0.5 \times 10⁶) and CD4⁺CD25⁺ (0.08–0.1 \times 10⁶) cells were resuspended in 100 μ l of PBS and injected i.v. into syngeneic RAG2-KO hosts. Recipient mice were sacrificed 8–10 wk later, and spleens were harvested. Splenocytes were counted by the trypan blue exclusion method, stained with FITC-coupled anti-CD4 mAb, and analyzed by flow cytometry. Total T cell counts were obtained as described above.

Cytokine analysis

WT and CTLA-4-Tg T cells enriched by negative selection were plated on flat-bottom 96-well plates (5 \times 10⁴/well) in the presence of soluble anti-CD3 mAb (10 mg/ml) and T-depleted irradiated WT splenocytes (2 \times 10⁵). Alternatively, plates were coated overnight at 4°C with anti-CD3 (10 μ g/ml in 50 mM Tris (pH 9.4)) and washed twice with complete medium before the addition of T cells. Supernatants were harvested 24 h after exposure of T cells to anti-CD3 mAb, and concentration of cytokines in each sample was detected by ELISA using Ab pairs as instructed by the manufacturer (BD Pharmingen). Absorbance was detected in a 96-well spectrophotometer (μ Quant; Bio-Tek Instruments, Winooski, VT), and data were analyzed using KC4 software (Bio-Tek Instruments) by comparison to a standard curve generated using recombinant cytokines at known concentrations.

Proliferation assays

Splenocytes (2 \times 10⁵/well) from CD28-KO and CTLA-4-Tg/CD28-KO mice were incubated with various concentrations of anti-CD3 mAb (27) in the presence or absence of recombinant human IL-2 (100 U/ml). Plates were pulsed with [³H]thymidine (1 μ Ci/well) for the last 6–8 h of a 48- or 72-h culture, as indicated in the text. [³H]Thymidine incorporation was measured using a TopCount NXT microplate scintillation and luminescence counter with TopCount NXT software (Packard BioScience, Meriden, CT).

Serum isotype determination

Mice between 7 and 16 wk of age were bled by retro-orbital puncture using nonheparinized capillary tubes, and serum concentration of IgG1 and IgE was determined by ELISA, according to the guidelines of the manufacturer (BD Pharmingen). Absorbance was detected in a 96-well spectrophotometer (μ Quant; Bio-Tek Instruments), and data were analyzed using KC4 software (Bio-Tek Instruments) by comparison to a standard curve generated using recombinant IgE and IgG1 at known concentrations.

Blood hemoglobin levels

Mice were bled by retro-orbital puncture using heparinized capillary tubes. Blood was analyzed for hematological composition at the University of Chicago Animal Resource Center.

Histology

Colons harvested from 16-wk-old WT, IL-2-KO, and CTLA-4-Tg/IL-2-KO mice were frozen in liquid nitrogen. The samples were sliced into 6- μ m-thick sections at -20°C. The slides were immersed briefly in hematoxylin for counterstaining and evaluated under light microscopy, by a pathologist blinded to the genotype of the mice.

Survival

Mice were examined three times per week, and animals were sacrificed when moribund, or when weight loss reached a 35% difference with control littermates, or when rectal prolapse was associated with a >10% weight loss.

Statistical analysis

Statistical analyses were performed using Student's *t* test.

Results

CTLA-4 is expressed in T cells from CTLA-4-Tg/IL-2-KO mice

We have previously reported that IL-2-deficient T cells are defective in their capacity to up-regulate CTLA-4 upon T cell activation (13). This may be because IL-2-deficient T cells have defects in TCR-mediated proliferation (data not shown), because IL-2 normally promotes cell cycle progression. Indeed, both cell cycle inhibitors and anti-IL-2 Abs prevent CTLA-4 expression in activated WT T cells, and IL-2 blockade also prevents T cell proliferation (13). To determine whether correction of the defect in CTLA-4 expression would prevent the disease in IL-2-deficient mice, we generated CTLA-4-Tg/IL-2-KO mice in which the *CTLA-4* transgene is expressed on T cells. To determine the spontaneous levels of CTLA-4 expression, permeabilized splenocytes from littermates were examined by flow cytometry. CD4⁺ and CD8⁺ T cells from both CTLA-4-Tg/IL-2^{+/-} and CTLA-4-Tg/IL-2-KO expressed significant levels of total CTLA-4, whereas levels in unstimulated IL-2^{+/-} and IL-2-KO T cells were nearly undetectable (Fig. 1A).

To compare the levels of CTLA-4 expression after activation, splenocytes were stimulated with anti-CD3 mAb for 72 h. Consistent with our published observation (13), little total CTLA-4 was detected in activated CD4⁺ and CD8⁺ IL-2-KO T cells when compared with T cells from IL-2-expressing controls (Fig. 1B; expression of CTLA-4 in activated IL-2^{+/-} T cells was similar to that in WT T cells, data not shown). In contrast, T cells from CTLA-4-Tg/IL-2-KO mice expressed high levels of CTLA-4 (Fig. 1B). Similarly to previous results obtained with C57BL/6 T cells (13), surface expression of CTLA-4 was barely detectable on activated nonpermeabilized T cells from all four strains (data not shown), suggesting that the ratio of intracellular to surface CTLA-4 expression induced by the transgene was similar to that of

endogenous CTLA-4. Together, these results indicate that the *CTLA-4* transgene bypasses the requirement for IL-2 signaling for CTLA-4 up-regulation and results in expression of CTLA-4 in IL-2-deficient T cells.

Forced expression of CTLA-4 prevents lymphoaccumulation in IL-2-deficient mice

To determine whether transgenic expression of CTLA-4 affected progressive lymphoaccumulation in IL-2-deficient mice, animals were sacrificed at different time points, and spleen weight and T cell composition were analyzed. IL-2-deficient mice developed severe splenomegaly with an average 8-fold increase in spleen weight when compared with WT mice at 16 wk of age (Fig. 2A). Remarkably, the weight of the spleen in 7- and 16-wk-old CTLA-4-Tg/IL-2-KO mice was dramatically lower than that in age-matched IL-2-KO mice, and was similar to that in WT mice, indicating that expression of the *CTLA-4* transgene prevented splenomegaly. Splenic T cell subpopulations were then analyzed by flow cytometry. Spleens from IL-2-KO mice displayed progressive accumulation of both CD4⁺ and CD8⁺ T cells when compared with spleens from WT littermates, resulting in a 3-fold increase in T cell numbers at 16 wk of age (Fig. 2B). In sharp contrast, CTLA-4-Tg/IL-2-KO mice had normal numbers of splenic T cells at both time points. Similar results were observed when lymph nodes were analyzed (data not shown). The large proportion of non-T cells in IL-2-deficient spleens that presumably makes up for the further increase in spleen weight was found to be granulocytes (GR1⁺; data not shown), reflecting extramedullary hemopoiesis, as IL-2-deficient mice develop severe acute hemolytic anemia (AHA) (see Fig. 8). Thus, expression of the *CTLA-4*

FIGURE 1. Transgene-controlled CTLA-4 induces CTLA-4 expression in IL-2-deficient T cells. Splenocytes from IL-2^{+/-}, CTLA-4-Tg/IL-2^{+/-}, IL-2-KO, and CTLA-4-Tg/IL-2-KO littermates were analyzed by flow cytometry either on day 0 (A) or 72 h after stimulation with soluble anti-CD3 mAb. Permeabilized splenocytes were stained with FITC-coupled anti-CD4 or anti-CD8 mAbs and with PE-coupled control hamster IgG (light histogram) or anti-CTLA-4 mAb (bold histogram). Events were gated on CD4⁺ (left half) or CD8⁺ (right half) cells, and PE relative fluorescence was displayed as a histogram. Numbers in the plots represent the mean fluorescence intensity of CTLA-4 expression in each cell type and the percentage of T cells that are positive for CTLA-4 expression.

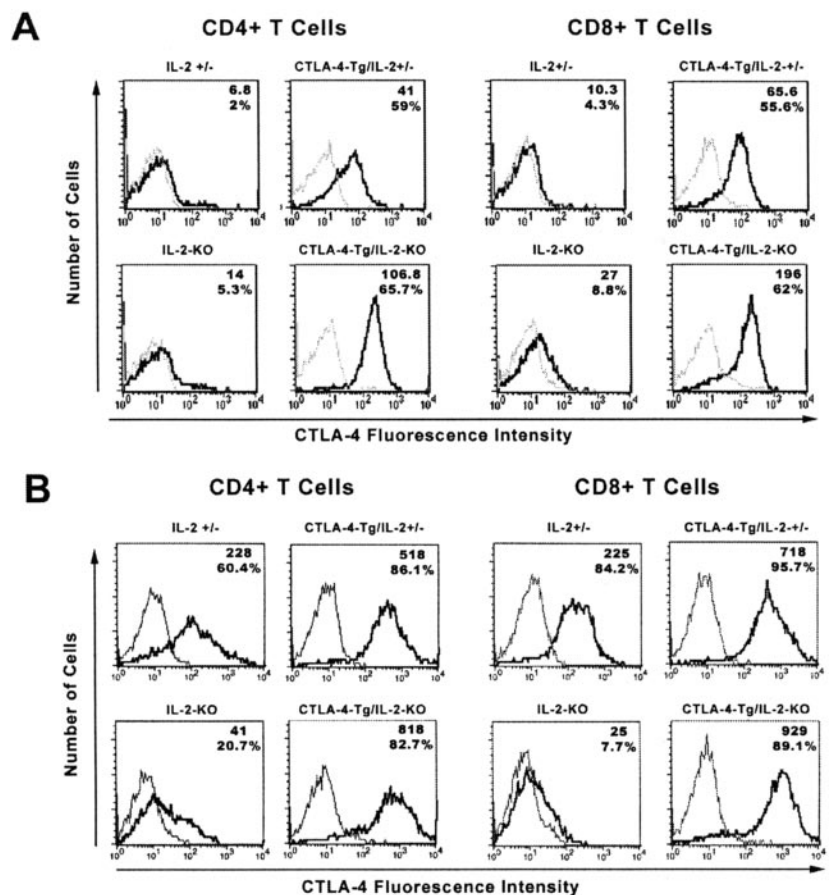
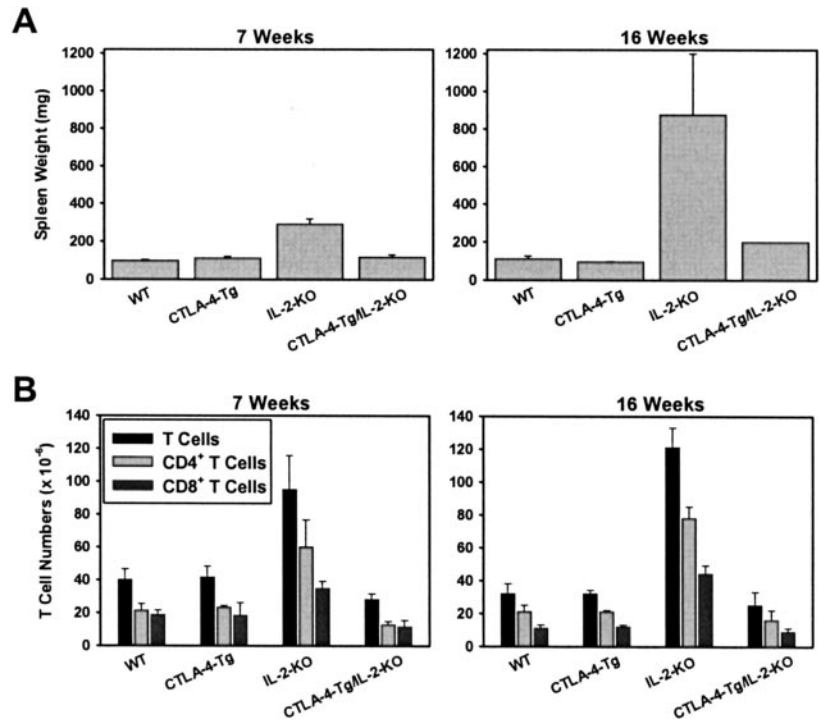


FIGURE 2. Expression of the *CTLA-4* transgene prevents progressive lymphoaccumulation in IL-2-deficient mice. *A*, Spleens were harvested from 7- and 16-wk-old littermates and weighed. Results are represented as the mean \pm SD of three to four mice per group from three independent experiments. *B*, Splenocytes from *A* were processed into single-cell suspensions. Live cells were counted by the trypan blue exclusion method. Cells were stained with FITC-coupled anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry. The percentage of T cell subsets was multiplied by the total cell counts to obtain the number of CD4⁺ and CD8⁺ T cells in the spleen.



transgene prevented T cell lymphoaccumulation even in older IL-2-KO mice.

Transgenic expression of CTLA-4 reduces the proliferation of T cells in vivo

The prevention of progressive T cell lymphoaccumulation by overexpression of CTLA-4 in T cells could be due either to inhibition of T cell proliferation or to induction of T cell death. To determine whether expression of the *CTLA-4* transgene prevented proliferation, CD4⁺ and CD8⁺ T cells were purified from the spleen of the different littermates and immediately analyzed by intracellular PI staining for the proportion of cells at each stage of the cell cycle. As shown in Fig. 3, both CD4⁺ and CD8⁺ IL-2-KO T cells had a marked increase in the percentage of cells in the S/G₂/M phases of the cell cycle when compared with WT cells. Expression of the *CTLA-4* transgene resulted in a dramatic reduction in the proportion of cycling IL-2-KO T cells. No significant increase in the percentage of subdiploid cells suggestive of increased T cell death

was observed, although it is possible that apoptotic cells are cleared in vivo too rapidly to allow for their detection ex vivo. When BrdU incorporation in vivo was analyzed, a similar reduction in the number of proliferating T cells was observed in CTLA-4-Tg/IL-2-KO mice when compared with IL-2-KO mice (data not shown). These results indicate that forced expression of CTLA-4 reduces the proliferation of IL-2-deficient T cells.

Forced expression of CTLA-4 does not restore normal numbers of CD4⁺CD25⁺ T cells in IL-2-deficient mice

Because endogenous CTLA-4 is expressed both in activated T cells as well as in CD4⁺CD25⁺ Treg, possible hypotheses to explain correction of the lymphoproliferation in IL-2-KO mice by transgenic expression of CTLA-4 included inhibition of conventional T cell responses and/or increase in the numbers or function of Treg. In this study, we have confirmed that most peripheral IL-2-KO T cells are defective in CTLA-4 expression after activation (see Fig. 1). To determine whether Treg from IL-2-deficient

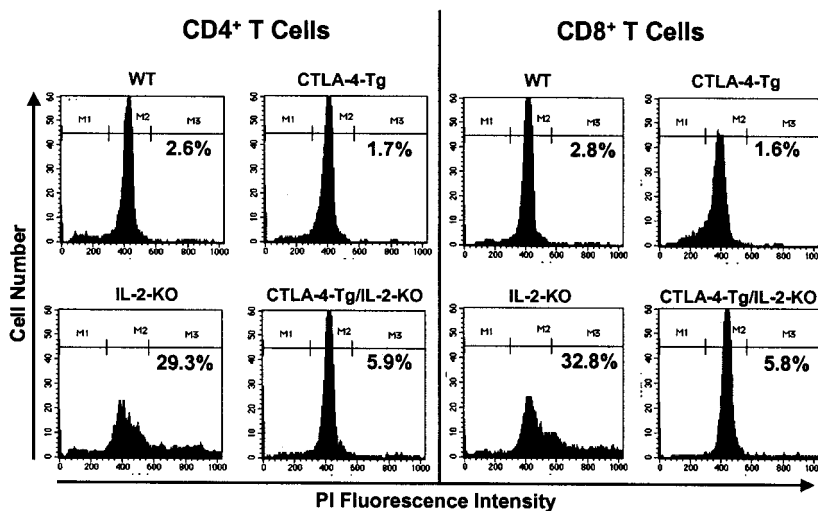
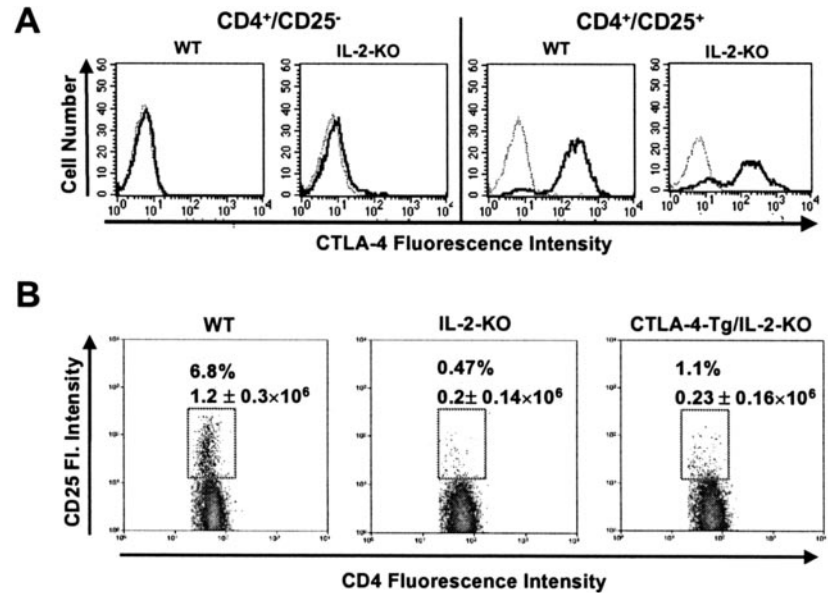


FIGURE 3. Transgenic expression of CTLA-4 reduces the percentage of T cells in the G₂/S/M phases of the cell cycle. Purified splenic CD4⁺ and CD8⁺ T cells were immediately fixed, permeabilized, stained with PI, and analyzed by flow cytometry. The numbers inside the plots represent the percentage of T cells in G₂/S/M phases of the cell cycle. This result is representative of at least three independent experiments.

FIGURE 4. Transgenic expression of CTLA-4 does not rescue the numbers of CD4⁺CD25⁺ cells in IL-2-KO mice. **A**, CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were sorted from WT and IL-2-KO splenocytes. Cells were then stained with PE-coupled control hamster IgG (light histogram) or anti-CTLA-4 mAb (bold histogram) and analyzed by flow cytometry. **B**, Splenocytes from WT, IL-2-KO, and CTLA-4-Tg/IL-2-KO mice were stained with anti-CD4-FITC and control hamster IgG or anti-CD25-4-PE, and analyzed by flow cytometry. Events were gated on CD4⁺ cells and displayed as a dot plot. Numbers in the plots represent the percentage of CD4⁺CD25⁺ cells within the CD4⁺ population as well as the total number of CD4⁺CD25⁺ cells in the spleen (the mean ± SD of three mice per group at 7–16 wk of age).



mice are also defective in constitutive expression of CTLA-4, WT and IL-2-KO CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were sorted and stained with anti-CTLA-4 mAb. As expected, no CTLA-4 expression was detected in CD4⁺CD25⁻ T cells in the absence of TCR stimulation (Fig. 4A, two left panels). Surprisingly, most IL-2-KO CD4⁺CD25⁺ T cells expressed similar constitutive levels of CTLA-4 as WT Treg (Fig. 4A, two right panels), suggesting that IL-2 is not necessary for the constitutive expression of CTLA-4 in this T cell subset. Next, we investigated whether overexpression of CTLA-4 rescued the numbers of Treg in IL-2-KO mice. When the percentage and total number of CD4⁺CD25⁺ cells were analyzed, we found that IL-2-deficient mice had a 6-fold reduction in the total number of CD4⁺CD25⁺ cells when compared with WT mice (Fig. 4B). Similar numbers were obtained from CTLA-4-Tg/IL-2-KO mice, indicating that forced expression of CTLA-4 did not increase the proportion or total number of CD4⁺ cells that express CD25 (Fig. 4B).

Forced expression of CTLA-4 does not restore the function of Treg in IL-2-deficient conditions

Next, we investigated whether forced expression of CTLA-4 could restore the function of IL-2-KO Treg. This was a possible explanation, because we have recently shown that Treg stimulated with anti-CD3 for 24 h up-regulate CTLA-4 expression above their constitutive level and that CTLA-4 in these activated Treg is responsible for their higher regulatory capacity (28). Thus, it was conceivable that, by enhancing the level of CTLA-4 expression in IL-2-KO Treg by means of the transgene, their suppressor function may also be increased. It has been shown that bulk IL-2-deficient CD4⁺ cells have suppressor activity in vivo when transferred into IL-2-sufficient mice, suggesting that IL-2-deficient Treg do have suppressor function when the responding T cells make IL-2 (29). Thus, to compare the suppressor capacity of IL-2-KO and CTLA-4-Tg/IL-2-KO Treg, we used IL-2-sufficient WT CD4⁺CD25⁻ cells as responders. As shown in Fig. 5A, proliferation of WT responder cells was reduced in a dose-dependent manner by increasing numbers of CD4⁺CD25⁺ cells, whether from WT, IL-2-KO, or CTLA-4-Tg/IL-2-KO mice, indicating that increased expression of CTLA-4 did not enhance the suppressor capacity of IL-2-KO Treg on a per-cell basis. Furthermore, CD25⁻ conventional cells isolated from IL-2-KO mice were much less effective

at inhibiting WT T cell responses, even though, like Treg, they do not make IL-2 and do not proliferate to anti-CD3 mAb in vitro in the absence of exogenous IL-2. This level of inhibition by conventional IL-2-KO T cells was probably due to competition for

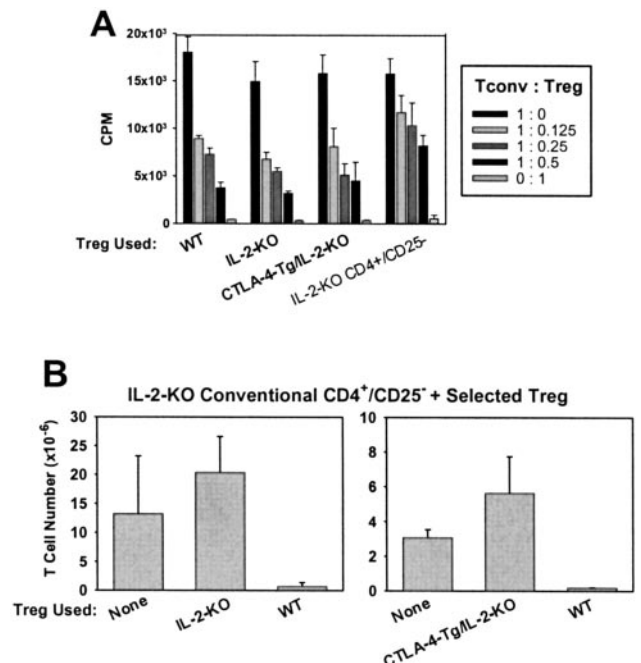


FIGURE 5. Transgenic expression of CTLA-4 does not restore the suppressor function of CD4⁺CD25⁺ cells in vitro or in vivo. CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were sorted from WT, IL-2-KO, and CTLA-4-Tg/IL-2-KO splenocytes. **A**, WT CD4⁺CD25⁻ cells were incubated with anti-CD3, T-depleted irradiated syngeneic splenocytes, in the presence or absence of various numbers of either CD4⁺CD25⁺ cells (Treg) from the different genotypes, or conventional CD4⁺CD25⁻ IL-2-KO cells. [³H]Thymidine incorporation was measured at 72 h. **B**, RAG2-KO recipients were injected i.v. with conventional CD4⁺CD25⁻ IL-2-KO cells either alone (*n* = 8, left panel; *n* = 4, right panel), or with CD4⁺CD25⁺ cells (Treg) from IL-2-KO mice (*n* = 3, left panel), WT mice (*n* = 4, left panel; *n* = 2, right panel), or CTLA-4-Tg/IL-2-KO mice (*n* = 2, right panel). Recipient mice were sacrificed after 8 wk, and splenocytes were analyzed by flow cytometry.

IL-2 or anti-CD3 mAb, and not to conventional Treg contaminating the CD25⁻ population, because levels of FoxP3 mRNA expression were high in CD25⁺ WT and IL-2-KO and very low in CD25⁻ WT and IL-2-KO (data not shown). The higher level of inhibition of WT T cells by CD25⁺ IL-2-KO T cells indicated that inhibition by IL-2-KO Treg was not just due to competition for IL-2 or anti-CD3 mAb and suggested that most of the suppression in IL-2-KO CD4⁺ cells resided within the CD4⁺CD25⁺ population.

There have been some discrepancies between the properties of Treg in vitro and in vivo. For instance, Treg do not proliferate in vitro in the absence of exogenous IL-2 but have been shown to expand in vivo. In addition, it was still possible that expression of the *CTLA-4* transgene could restore the suppressor function of IL-2-KO Treg in an IL-2-deficient setting. To address both points, we developed an adoptive transfer system. Transfer of CD4⁺ cells into RAG-deficient mice is known to lead to lymphoaccumulation (30). CD4⁺CD25⁻ IL-2-KO cells were transferred into syngeneic RAG2-KO mice in the presence or absence of Treg from the various genotypes, and recipient animals were analyzed after 8 wk. Transfer of WT Treg very effectively limited the long-term expansion of splenic IL-2-KO CD4⁺ cells (Fig. 5B, left panel). This is not likely due to secretion of IL-2 by WT Treg, because CD4⁺CD25⁺ cells are anergic and do not produce IL-2. In contrast, cotransfer of IL-2-KO Treg cells did not reduce, but enhanced lymphoaccumulation, indicating that IL-2-KO CD4⁺CD25⁺ do not have suppressor function in vivo in an IL-2-deficient setting. Similarly, Treg from CTLA-4-Tg/IL-2-KO mice did not prevent, but augmented accumulation of IL-2-KO CD4⁺ cells in recipient RAG-KO mice (Fig. 5B, right panel). Thus, transgenic overexpression of CTLA-4 did not restore the suppressor function of CD4⁺CD25⁺ IL-2-KO cells in IL-2-deficient conditions in vivo.

The inhibitory effect of the CTLA-4 transgene depends on the presence of APCs

If expression of the *CTLA-4* transgene was not rescuing either the number or the function of IL-2-KO Treg in vivo, it seemed plausible that the prevention of lymphoproliferation was due to direct inhibition of expansion of conventional T cells by the transgene. To exclude that the reduced response by CTLA-4-Tg T cells was due to a developmental defect leading to an intrinsically reduced T cell activation capacity, T cells were purified and stimulated in vitro in the presence of soluble anti-CD3 and syngeneic APCs or with immobilized anti-CD3 and anti-CD28 mAbs. Supernatants were collected at 24 h and analyzed by ELISA for cytokine content. As shown in Fig. 6, CTLA-4-Tg T cells produced reduced IL-2 and IFN- γ compared with WT T cells when stimulated in the presence of APCs. In contrast, cytokine production by WT and CTLA-4-Tg T cells was similar when TCR stimulation occurred in the absence of APCs. This result indicates that the intrinsic activation capacity of CTLA-4-Tg T cells is similar to that of WT T cells. Thus, reduced responses of CTLA-4-Tg conventional T cells are likely secondary to the binding of CTLA-4 to ligands on APCs.

The inhibitory effect of the CTLA-4 transgene occurs, at least in part, via a CD28-independent mechanism

There are at least two possible mechanisms by which the *CTLA-4* transgene could inhibit the responses of conventional T cells in an APC-dependent manner. First, constitutive expression of CTLA-4 could very effectively prevent engagement of B7 family members by CD28, because it has a higher affinity than CD28 for its ligands. Second, engagement of CTLA-4 could activate intracellular biochemical pathways resulting in inhibition of TCR-mediated sig-

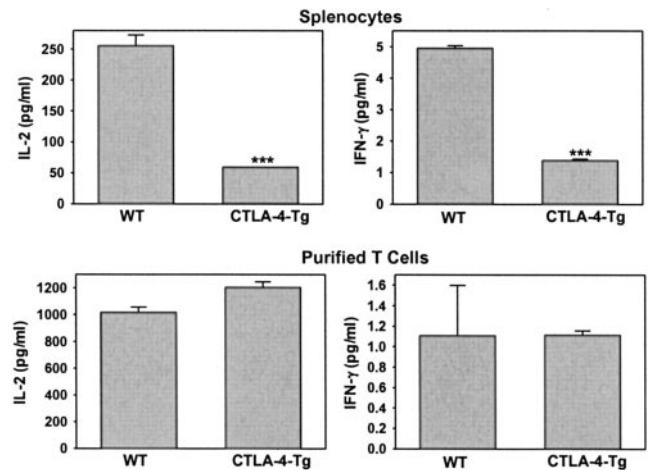


FIGURE 6. The inhibitory effect mediated by the *CTLA-4* transgene depends on the presence of APCs. WT and CTLA-4-Tg T cells were purified from splenocytes and incubated with either soluble anti-CD3 mAb (10 μ g/ml) in the presence of syngeneic APCs or plate-bound anti-CD3 mAb (10 μ g/ml). Supernatants were collected at 24 h, and cytokine content was analyzed by ELISA. The plots represent the mean + SD of triplicate determinations. ***, $p < 0.001$.

nals. To determine whether the *CTLA-4* transgene could inhibit T cell responses in the absence of CD28 expression, CTLA-4-Tg mice were crossed with CD28-deficient mice. CD4⁺ and CD8⁺ T cells were purified from resulting CD28-KO and CTLA-4-Tg/CD28-KO littermates, stained with CFSE, and stimulated in vitro with anti-CD3 mAb in the presence of syngeneic APCs. Fewer CTLA-4-Tg/CD28-KO T cells underwent cell division than CD28-KO T cells at all time points examined (Fig. 7A), indicating that the *CTLA-4* transgene can inhibit T cell responses independently of CD28 expression.

To determine whether reduced T cell responses in vitro were due to reduced IL-2 production, splenocytes from CD28-KO and CTLA-4-Tg/CD28-KO mice were stimulated with soluble anti-CD3 mAb in the presence or absence of saturating amounts of IL-2 (100 U/ml), and [³H]thymidine incorporation was measured at different time points. As shown in Fig. 7B, although the presence of IL-2 promoted much greater [³H]thymidine incorporation overall, reduced responses by CTLA-4-Tg/CD28-KO T cells were still observed when compared with CD28-KO T cells. Thus, the reduced T cell response by CTLA-4-Tg T cells is not solely due to a decrease in production of the growth factor IL-2.

Forced expression of CTLA-4 prevents AHA in IL-2-deficient mice

As a consequence of uncontrolled T cell activation differentiation and proliferation, IL-2-deficient mice develop B cell-mediated AHA (15). In these mice, AHA correlates with increased serum titers of Abs (mostly IgE and IgG1). If the *CTLA-4* transgene is preventing proliferation of conventional T cells, one may expect reduced B cell help and prevention of AHA in CTLA-4-Tg/IL-2-KO mice. To investigate the effect of forced expression of CTLA-4 on AHA, serum from 7- to 16-wk-old mice was collected. Expression of the *CTLA-4* transgene in IL-2-KO mice significantly reduced the levels of IgE and IgG1 when compared with those in IL-2-deficient mice (Fig. 8A). Consistent with this reduction and with the normal spleen weight that suggested complete prevention of extramedullary hemopoiesis, CTLA-4-Tg/IL-2-KO mice did not develop anemia (Fig. 8B).

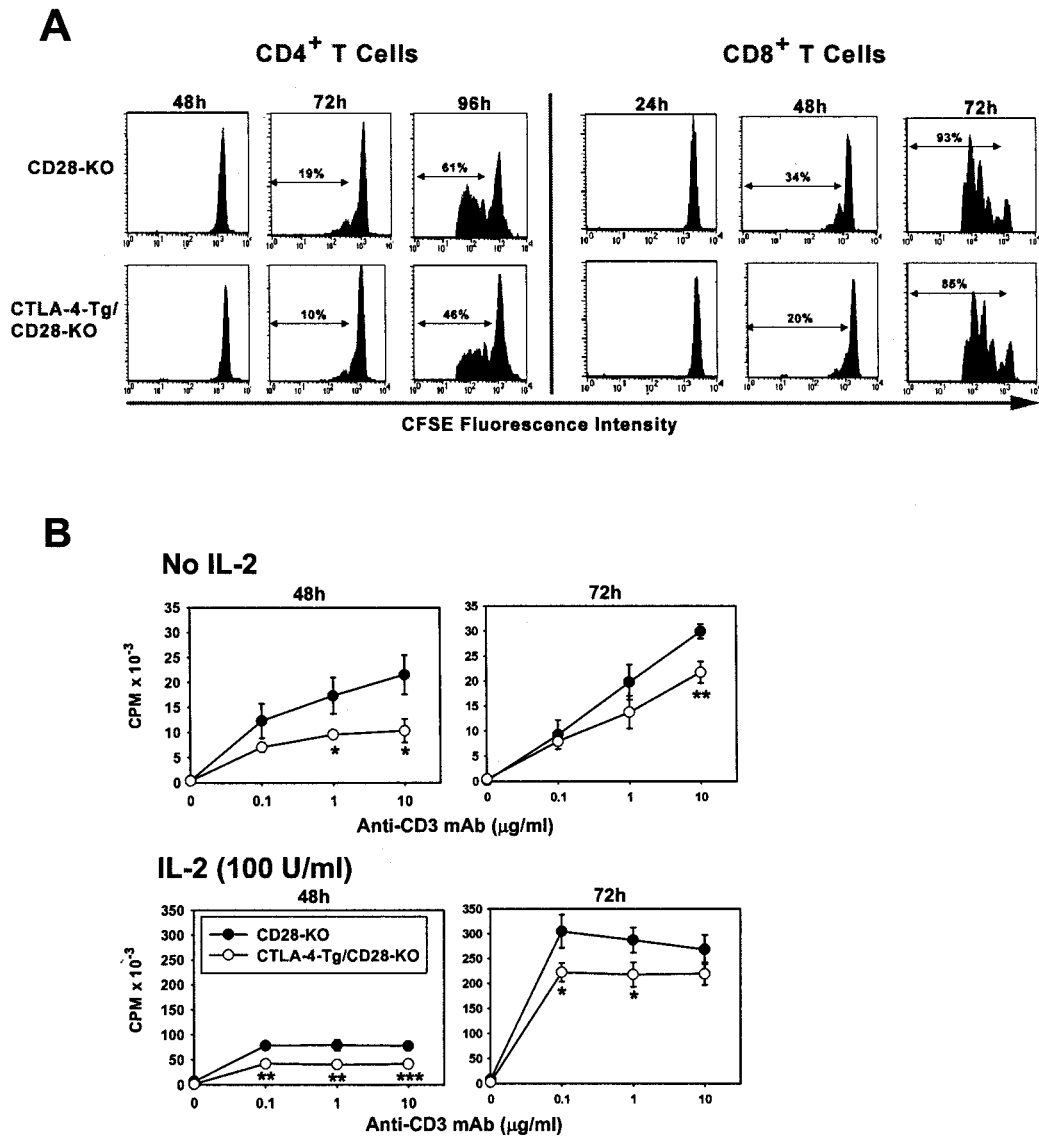


FIGURE 7. The inhibitory effect of the *CTLA-4* transgene is, at least in part, independent of CD28 expression. *A*, T cells were purified from CD28-KO and CTLA-4-Tg/CD28-KO splenocytes, labeled with CFSE, incubated in the presence of anti-CD3 mAb (10 μg/ml) and syngeneic APCs, and analyzed by flow cytometry at different time points. Events were gated on CD4⁺ or CD8⁺ cells, and CFSE fluorescence was displayed as histograms. The numbers in the plots represent the percentages of CD4⁺ or CD8⁺ cells that have undergone at least one cell division. This result is representative of two independent experiments. *B*, Splenocytes from CD28-KO and CTLA-4-Tg/CD28-KO mice were incubated in the presence of anti-CD3 mAb either in the absence (*top panels*) or in the presence (*bottom panels*) of large amounts of IL-2 (100 U/ml). Incorporation of [³H]thymidine for the last 6 h of a 48- or 72-h culture was assessed. Results represent the mean + SD of triplicate determinations. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

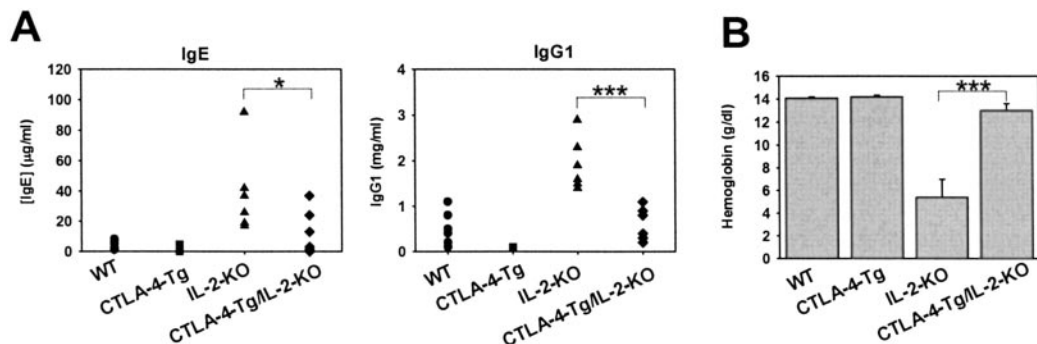


FIGURE 8. Transgenic expression of CTLA-4 prevents AHA in IL-2-KO mice. *A*, Seven- to 16-wk-old littermates (six mice in each group) were bled by retro-orbital puncture, and serum was analyzed for IgG1 and IgE content by ELISA. *, *p* < 0.05; ***, *p* < 0.001. *B*, Heparinized blood from the same animals was analyzed for hemoglobin content. Results represent the mean ± SD of six samples per group.

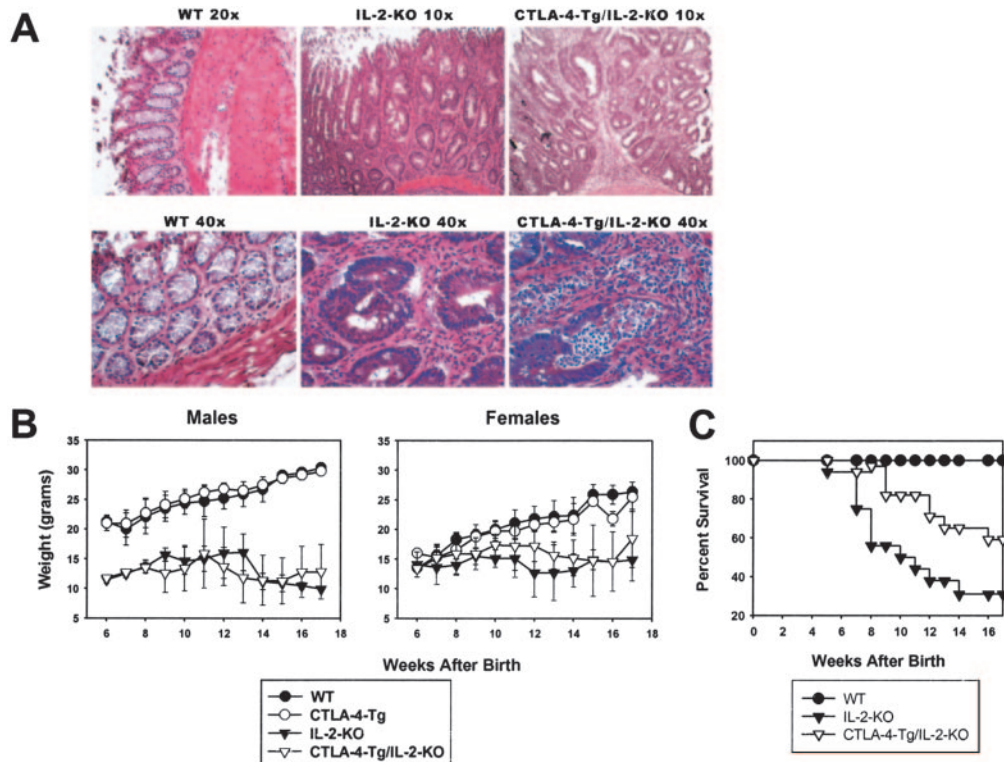


FIGURE 9. Forced expression of CTLA-4 does not prevent IBD but moderately prolongs survival of IL-2-KO mice. *A*, Colons from 16-wk-old littermates were examined by histology after H&E counterstaining. Note that, in the *top panels*, the WT colon is displayed at a $\times 20$ magnification, whereas those from IL-2-KO and CTLA-4-Tg/IL-2-KO mice are shown at a $\times 10$ magnification to allow display of all structures despite significant edema. *Bottom panels* are all displayed at $\times 40$ magnification. *B*, The weight of male and female mice of the different genotypes was recorded over time. *C*, The survival of 16 IL-2-KO and 18 CTLA-4-Tg/IL-2-KO mice was recorded over time.

In sharp contrast, expression of the transgene did not reduce the incidence or severity of inflammatory bowel disease (IBD) in IL-2-deficient mice (Fig. 9A). Compared with the normal colon of a WT mouse (*left panel*), representative sections of the colon from both IL-2-KO (*middle panel*) and CTLA-4-Tg/IL-2-KO (*right panel*) mice revealed similar marked edema, hyperplastic crypts, and increased mononuclear inflammatory cell infiltrates in the lamina propria. As an independent indicator of IBD, the weight of male and female mice was recorded over time. IL-2-KO mice developed progressive wasting. Consistent with colon histology, expression of the *CTLA-4* transgene did not prevent weight loss in IL-2-KO mice (Fig. 9B).

In keeping with lack of AHA but persistent IBD, CTLA-4-Tg/IL-2-KO mice had a significant ($p < 0.05$), but not dramatic increase in their life span when compared with IL-2-KO mice (Fig. 9C). Together, these results indicate that restoring CTLA-4 expression levels on T cells leads to prevention of lymphoproliferation and AHA, but not of IBD, and suggest that reduced CTLA-4 expression on T cells may play a role in the pathogenesis of AHA and not of IBD.

Discussion

Because IL-2-mediated signals are critical for the up-regulation of CTLA-4 on conventional T cells following T cell activation, we hypothesized that reduced CTLA-4 expression in IL-2-deficient T cells participates in the pathogenesis of the lymphoproliferative disease of IL-2-KO mice. We found that forced expression of CTLA-4 in IL-2-KO T cells prevented the progressive lymphoaccumulation and development of severe AHA that occur in these mice. In contrast, no reduction in the severity of IBD was observed, indicating that, despite arising from a common defect, the

mechanisms leading to IBD and AHA in IL-2-deficient animals differ in their dependency on CTLA-4 levels. In addition, reduced lymphoaccumulation was traced to a predominant effect of the *CTLA-4* transgene on conventional rather than regulatory T cells. Our results suggest that expression of CTLA-4 on conventional T cells plays a role in maintaining normal T cell homeostasis in a manner independent of Treg.

Establishing a causal link between reduced CTLA-4 expression and lymphoaccumulation in IL-2-deficient mice is challenging, because it is possible that overexpression of any molecule that induces cell cycle arrest or apoptosis in T cells may prevent lymphoaccumulation in these mice. We have shown a correlation between low levels of CTLA-4 expression in IL-2-KO T cells and development of lymphoproliferation. We have established that IL-2 is necessary for optimal CTLA-4 up-regulation (13). In this study, we demonstrate that restoring CTLA-4 expression is sufficient to prevent the lymphoproliferation and AHA, but not IBD, in IL-2-deficient mice. Other investigators have shown that transfer of wild-type Treg at birth is sufficient to prevent lymphoproliferation, AHA, and IBD in IL-2 β R-deficient mice (24). However, this adoptive transfer did not prevent disease in IL-2-deficient mice, because IL-2 produced by the recipient was necessary for engraftment and/or expansion of the transferred Treg (24). In this study, we show that even in the absence of functional Treg and of IL-2 in the recipient, overexpression of CTLA-4 is sufficient to prevent lymphoproliferation and AHA.

The levels of CTLA-4 expressed in activated CTLA-4-Tg T cells were 2- to 3-fold higher than those in WT cells. In addition, in contrast to WT T cells, resting CTLA-4-Tg T cells also expressed CTLA-4. We cannot exclude the possibility that prevention of lymphoproliferation and AHA is due to supraphysiological

levels of CTLA-4 expression rather than to restoration of normal CTLA-4 expression in IL-2-KO T cells. However, our results indicate that overexpressing CTLA-4 in T cells in an autoimmune disease in which reduced CTLA-4 expression is identified is sufficient to prevent multiple aspects of this disease.

The mechanisms by which endogenous CTLA-4 inhibits T cell responses are several. First, CTLA-4 can scavenge B7 family members away from CD28, therefore reducing positive costimulation of T cells (12, 25). However, this is not likely to be the mechanism by which transgenic CTLA-4 prevents the lymphoproliferative disease in IL-2-deficient mice, because CD28/IL-2-double-deficient mice have been generated and shown to display similar lymphoproliferation as IL-2-deficient controls (31). Second, CTLA-4 has been shown to inhibit T cell responses even in CD28-deficient T cells, suggesting direct inhibition of TCR signals (32, 33). A proposed mode of action of CTLA-4 is that the cytoplasmic tail of CTLA-4 associates with Src homology 2 domain-containing tyrosine phosphatase and dephosphorylates molecules important for proximal events of TCR signaling (34). In this study, we show that overexpression of the *CTLA-4* transgene can also inhibit T cells independently of CD28 expression. Finally, CTLA-4 may be necessary for the function of Treg, although this does not appear to be the case in all model systems. Initial mouse studies suggested that CTLA-4 expressed on Treg is necessary for the suppressor function of these cells in vitro (35) and in vivo (36). However, CTLA-4-deficient CD4⁺CD25⁺ mouse T cells retain suppressor function in vitro (35), and human Treg appear to function in a CTLA-4-independent manner (37, 38). Our results indicate that transgenic overexpression of CTLA-4 does not restore the development, survival, or function of IL-2-KO CD4⁺CD25⁺ Treg, but the *CTLA-4* transgene affects the function of conventional T cells.

In contrast to the other manifestations of disease in IL-2-deficient mice, IBD was not prevented by expression of the *CTLA-4* transgene. This may be because some consequences of lack of IL-2 are independent of reduced CTLA-4 expression. In support of this hypothesis, Ashcroft et al. (39) have recently reported that overproduction by IL-2-deficient CD4⁺ T cells of the ligand for the receptor activator of NF- κ B (RANKL), is responsible for the increased bone turnover and the IBD in IL-2-deficient mice, and blockade of the receptor activator of NF- κ B (RANK)/RANKL pathway using recombinant osteoprotegerin reduces colitis by decreasing the numbers of RANK⁺ dendritic cells in the colon. In contrast, blockade of RANK/RANKL interactions did not affect the lymphoaccumulation or AHA in these mice. Thus, IL-2-deficient T cells have reduced CTLA-4 expression but increased production of RANKL, and these two defects seem responsible for lymphoaccumulation and AHA, or osteopenia and IBD, respectively. Consistent with the lack of correlation between reduced CTLA-4 expression and IBD, CTLA-4-deficient mice do not develop IBD (M.-L. Alegre, unpublished observation; data not shown), and CTLA-4 polymorphisms have not been associated with IBD in humans (40).

Several studies have implicated CTLA-4 polymorphisms in the development of various autoimmune diseases in humans, and we have reported that one of these polymorphisms, in exon 1 of the *CTLA-4* gene, correlates with reduced inhibitory function of the CTLA-4 protein (41). More recently, susceptibility to Grave's disease, autoimmune hypothyroidism, and type 1 diabetes has been correlated with reduced mRNA expression of a transcript encoding for a soluble form of CTLA-4 (42). Together, these studies indicate that both reduced expression and reduced function of CTLA-4 in T cells may favor the development of autoimmune diseases in humans. Our study suggests that reduced expression of CTLA-4 in IL-2-KO conventional T cells may be an additional previously

unrecognized mechanism that contributes to the development of lymphoaccumulation and autoimmunity in IL-2-deficient mice. Thus, restoring normal CTLA-4 expression in T cells may be a possible strategy to prevent or control clinical autoimmune diseases in which CTLA-4 expression is reduced.

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References

- Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* 14:233.
- Krueger, A., S. C. Fas, S. Baumann, and P. H. Krammer. 2003. The role of CD95 in the regulation of peripheral T-cell apoptosis. *Immunol. Rev.* 193:58.
- Okazaki, T., Y. Iwai, and T. Honjo. 2002. New regulatory co-receptors: inducible co-stimulator and PD-1. *Curr. Opin. Immunol.* 14:779.
- Watanabe, N., M. Gavrieli, J. R. Sedy, J. Yang, F. Fallarino, S. K. Loftin, M. A. Hurchla, N. Zimmerman, J. Sim, X. Zang, et al. 2003. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat. Immunol.* 8:8.
- Francois Bach, J. 2003. Regulatory T cells under scrutiny. *Nat. Rev. Immunol.* 3:189.
- Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356:314.
- Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541.
- Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science* 270:985.
- Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the *PD-1* gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11:141.
- Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291:319.
- Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 4:330.
- Carreno, B. M., F. Bennett, T. A. Chau, V. Ling, D. Luxenberg, J. Jussif, M. L. Baroja, and J. Madrenas. 2000. CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. *J. Immunol.* 165:1352.
- Alegre, M. L., P. J. Noel, B. J. Eisfelder, E. Chuang, M. R. Clark, S. L. Reiner, and C. B. Thompson. 1996. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. *J. Immunol.* 157:4762.
- Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2-deficient by gene targeting. *Nature* 352:621.
- Ma, A., M. Datta, E. Margosian, J. Chen, and I. Horak. 1995. T cells, but not B cells, are required for bowel inflammation in interleukin 2-deficient mice. *J. Exp. Med.* 182:1567.
- Willerford, D. M., J. Chen, J. A. Ferry, L. Davidson, A. Ma, and F. W. Alt. 1995. Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* 3:521.
- Suzuki, H., T. M. Kundig, C. Furlonger, A. Wakeham, E. Timms, T. Matsuyama, R. Schmits, J. J. L. Simard, P. S. Ohashi, H. Griesser, et al. 1995. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor β . *Science* 268:1472.
- Schimpl, A., I. Berberich, B. Kneitz, S. Kramer, B. Santner-Nanan, S. Wagner, M. Wolf, and T. Hunig. 2002. IL-2 and autoimmune disease. *Cytokine Growth Factor Rev.* 13:369.
- Lenardo, M. J. 1991. Interleukin-2 programs mouse α - β T lymphocytes for apoptosis. *Nature* 353:858.
- Refaeli, Y., L. Van Parijs, C. A. London, J. Tschopp, and A. K. Abbas. 1998. Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. *Immunity* 8:615.
- Papiernik, M., M. L. de Moraes, C. Pontoux, F. Vasseur, and C. Penit. 1998. Regulatory CD4 T cells: expression of IL-2R α chain, resistance to clonal deletion and IL-2 dependency. *Int. Immunol.* 10:371.
- Wolf, M., A. Schimpl, and T. Hunig. 2001. Control of T cell hyperactivation in IL-2-deficient mice by CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells: evidence for two distinct regulatory mechanisms. *Eur. J. Immunol.* 31:1637.
- Malek, T. R., B. O. Porter, E. K. Codias, P. Scibelli, and A. Yu. 2000. Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors. *J. Immunol.* 164:2905.

24. Malek, T. R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2R β -deficient mice: implications for the nonredundant function of IL-2. *Immunity* 17:167.
25. Masteller, E. L., E. Chuang, A. C. Mullen, S. L. Reiner, and C. B. Thompson. 2000. Structural analysis of CTLA-4 function in vivo. *J. Immunol.* 164:5319.
26. Alegre, M. L., H. Shiels, C. B. Thompson, and T. F. Gajewski. 1998. Expression and function of CTLA-4 in Th1 and Th2 cells. *J. Immunol.* 161:3347.
27. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* 84:1374.
28. Fallarino, F., U. Grohmann, K. W. Hwang, C. Orabona, C. Vacca, R. Bianchi, M. L. Belladonna, M. C. Fioretti, M. L. Alegre, and P. Puccetti. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nat. Immunol.* 26:26.
29. Furtado, G. C., M. A. De Lafaille, N. Kutchukhidze, and J. J. Lafaille. 2002. Interleukin-2 signaling is required for CD4⁺ regulatory T cell function. *J. Exp. Med.* 196:851.
30. Surh, C. D., and J. Sprent. 2002. Regulation of naive and memory T-cell homeostasis. *Microbes Infect.* 4:51.
31. Boone, D. L., T. Dassopoulos, J. P. Lodolce, S. Chai, M. Chien, and A. Ma. 2002. Interleukin-2-deficient mice develop colitis in the absence of CD28 costimulation. *Inflamm. Bowel Dis.* 8:35.
32. Lin, H., J. C. Rathmell, G. S. Gray, C. B. Thompson, J. M. Leiden, and M. L. Alegre. 1998. Cytotoxic T lymphocyte antigen 4 (CTLA4) blockade accelerates the acute rejection of cardiac allografts in CD28-deficient mice: CTLA4 can function independently of CD28. *J. Exp. Med.* 188:199.
33. Fallarino, F., P. E. Fields, and T. F. Gajewski. 1998. B7-1 engagement of cytotoxic T lymphocyte antigen 4 inhibits T cell activation in the absence of CD28. *J. Exp. Med.* 188:205.
34. Lee, K. M., E. Chuang, M. Griffin, R. Khattri, D. K. Hong, W. Zhang, D. Straus, L. E. Samelson, C. B. Thompson, and J. A. Bluestone. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science* 282:2263.
35. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25⁺CD4⁺ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192:303.
36. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192:295.
37. Levings, M. K., R. Sangregorio, and M. G. Roncarolo. 2001. Human CD25⁺CD4⁺ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J. Exp. Med.* 193:1295.
38. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A. H. Enk. 2001. Identification and functional characterization of human CD4⁺CD25⁺ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193:1285.
39. Ashcroft, A. J., S. M. Cruickshank, P. I. Croucher, M. J. Perry, S. Rollinson, J. M. Lippitt, J. A. Child, C. Dunstan, P. J. Felsburg, G. J. Morgan, and S. R. Carding. 2003. Colonic dendritic cells, intestinal inflammation, and T cell-mediated bone destruction are modulated by recombinant osteoprotegerin. *Immunity* 19:849.
40. Xia, B., J. B. Crusius, J. Wu, A. Zwiers, A. A. van Bodegraven, and A. S. Pena. 2002. *CTLA4* gene polymorphisms in Dutch and Chinese patients with inflammatory bowel disease. *Scand. J. Gastroenterol.* 37:1296.
41. Kouki, T., Y. Sawai, C. A. Gardine, M. E. Fisfalen, M. L. Alegre, and L. J. DeGroot. 2000. *CTLA-4* gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves' disease. *J. Immunol.* 165:6606.
42. Ueda, H., J. M. Howson, L. Esposito, J. Heward, H. Snook, G. Chamberlain, D. B. Rainbow, K. M. Hunter, A. N. Smith, G. Di Genova, et al. 2003. Association of the T-cell regulatory gene *CTLA4* with susceptibility to autoimmune disease. *Nature* 423:506.