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Lymphoproliferative Disorder in CTLA-4 Knockout Mice Is Characterized by CD28-Regulated Activation of Th2 Responses

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Mice lacking CTLA-4 die at an age of 2–3 wk due to massive lymphoproliferation, leading to lymphocytic infiltration and destruction of major organs. The onset of the lymphoproliferative disease can be delayed by treatment with murine CTLA4Ig (mCTLA4Ig), starting day 12 after birth. In this study, we have characterized the T cells present in CTLA-4-deficient mice before and after mCTLA4Ig treatment. The T cells present in CTLA-4-deficient mice express the activation markers, CD69 and IL-2R; down-regulate the lymphoid homing receptor, CD62L; proliferate spontaneously in vitro and cannot be costimulated with anti-CD28 mAb consistent with a hyperactivated state. The T cells from CTLA-4-deficient mice survive longer in culture correlating with higher expression of the survival factor, Bcl-xL, in these cells. Most significantly, the CD4+ T cell subset present in CTLA-4-deficient mice secretes high levels of IL-4 and IL-5 upon TCR activation. Treatment of CTLA-4-deficient mice treated with mCTLA4Ig reverses the activation and hyperproliferative phenotype of the CTLA-4-deficient T cells and restores the costimulatory activity of anti-CD28 mAb. Furthermore, T cells from mCTLA4Ig-treated mice are not skewed toward a Th2 cytokine phenotype. Thus, CTLA-4 regulates CD28-dependent peripheral activation of CD4+ T cells. This process results in apoptosis-resistant, CD4+ T cells with a predominantly Th2 phenotype that may be involved in the lethal phenotype in these animals. The Journal of Immunology, 1999, 162: 5784–5791.

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R.K. and J.A.A. have contributed equally to the study and should be considered co-first authors.

A.H.S. and J.A.B. are co-senior authors on this study.

3 Abbreviations used in this paper: mCTLA4Ig, murine CTLA4Ig; CD62L, CD62 ligand; CTLA-4KO, CTLA-4 knockout; LN, lymph node; NOD, nonobese diabetic; PI, propidium iodide; WT, wild type.

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as day 12 postbirth (after the initial appearance of activated T cells). The T cells isolated from mCTLA4Ig-treated animals were normal by all of the criteria tested. Thus, these studies support a model in which the autoimmune disease manifested in the absence of CTLA-4 is due to activation via a CD28/B7-dependent pathway leading to expansion of autodestructive Th2-type CD4+ T cells.

Materials and Methods

Animals and treatment

Mice deficient in CTLA-4 were generated as previously described (18) and bred onto the NOD background. Mice were kept in a specific-pathogen-free animal barrier facility at the University of Chicago (Chicago, IL). The F1 heterozygotes were bred to generate the CTLA-4KO animals. The mice were screened by PCR of tail DNA using the oligonucleotide primers for CTLA-4 (5'-ATGGTTGTGCTAGACCCATG and 3'-TTGGATTGTCGAGCTTACCT) and the neomycin resistance gene (5'-ATGGAACAAGATGGATTGCCAG and 3'-CGTCCAGTATCCGATGATC). Mice were treated with 100 μg of mCTLA4Ig given i.p. days 12, 15, and 18 after birth.

Flow cytometry

LN cells from wild type (WT) and CTLA-4KO mice were stained with various biotin- or FITC-coupled Abs to examine cell surface phenotype. Aliquots of 5 × 10^6 cells were incubated with 20 μl culture supernatant of 2.4G2 (rat anti-murine FcR) (24) mAb (to block FcR binding) and the relevant Abs in PBS-A buffer (PBS, 0.2% BSA, and 0.02% NaN3) for 30 min at 4°C. The cells were washed twice with PBS-A buffer and, if necessary, counterstained with PE-coupled streptavidin (Southern Biotechnologies, Birmingham, AL) for 15 min at 4°C, and washed once with PBS-A buffer before analysis. Stained cells (1 × 10^6) were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) using LYSIS II and WinMDI software packages (WinMDI software by Joseph Trotter; available from www.coloredmice.com). T cell subsets and the activation state of the cells were determined based on expression of various cell surface markers: CD3, CD4, CD8, Pgp-1 (CD44), Mel-14 (CD62L), IL-2R (CD25), CD69, and CD45R/B220, using directly conjugated mAbs (all purchased from PharMingen, San Diego, CA, or Boehringer Mannheim, Indianapolis, IN). For measuring viability, LN cells were stained as described for proliferation assays below. At appropriate time points, cells from three wells were harvested and pooled together. The cells were not washed before staining to minimize the loss of apoptotic or dead cells. A total of 10 μl of 0.1 mg/ml propidium iodide (PI) in PBS (Sigma, St. Louis, MO) was added just after analysis on a FACScan. All samples were run for a fixed time period (15 s). Cells staining PI<sup>−</sup> were considered to be the dead cells, and PI<sup>+</sup> cells were considered to be alive.

Bcl-x<sub>1</sub> staining

Aliquots of 1 × 10<sup>6</sup> LN cells were fixed with paraformaldehyde (1%; Polysciences, Warrington, PA) in PBS, for 10 min at room temperature, and washed once with 0.03% saponin (Sigma) in PBS. The cells were then permeabilized with 100 μl 0.3% saponin, blocked with goat serum (20 μl), and stained with culture supernatant (30 μl) of the anti-Bcl-x<sub>1</sub> mAb, 7B2, for 30 min at 4°C (7). The cells were washed once with 0.03% saponin and stained with FITC-coupled anti-mouse IgG3 (PharMingen) in 0.3% saponin for 30 min at 4°C. Before analysis on FACScan, the cells were washed twice with 0.03% saponin and once with PBS-A buffer. An isotype-matched control Ab was used for control staining.

Proliferation assays

LN cells were enriched for T cells by passage over nylon wool columns. MHC class II<sup>+</sup> cells were depleted further using a mixture of an anti-heat stable Ag (Jld) (25) and anti-I-A<sup>+</sup> (25-9-3) (26) culture supernatants plus rabbit complement (Pel-Freez, Brown Deer, WI). To enrich for CD4<sup>+</sup> T cells, the CD8<sup>+</sup> T cells were depleted with anti-CD8 mAb (3.155) (27); to enrich for CD8<sup>+</sup> cells, the CD4<sup>+</sup> T cells were depleted with anti-CD4 mAb (RL172.4) (28). T cell subset purity was evaluated by flow cytometry using anti-CD3, anti-CD4, or anti-CD8 mAbs. In all cases, the T cells used in these assay were >95% pure. T cells (2 × 10<sup>5</sup>) were stimulated with serial dilutions of anti-CD3ε (145-2C11) (29) with or without anti-CD28 (PV-1, 1 μg/ml) (30) and/or anti-IL-2 (25 U/ml). In experiments utilizing separated T cell subsets, 2 × 10<sup>5</sup> purified, irradiated, syngeneic APC were added to individual wells of a 96-well flat-bottom plate. Syngeneic feeder cells were depleted of T cells by incubation with anti-Thy-1.2 and rabbit complement. The cultures were incubated for 48 h at 37°C and pulsed with 1 μCi [H]<sup>3</sup>thymidine/well for the final 8 h of culture. The cultures were harvested using a Packard Filtermate 96-well harvester and analyzed on a Packard TopCount microplate scintillation counter (Packard Instrument, Meriden, CT). SEs for all experiments were routinely <20%.

Cytokine assays

Supernatants from triplicate cultures of activated T cells were harvested and combined at 24 h after stimulation for IL-2 and 48 h after stimulation for IL-4 and IFN-γ assays. IL-2 and IL-4 cytokines were measured using an ELISA (Endogen, Cambridge, MA). A murine IL-2 or IL-4 standard was used to quantitate cytokine levels in the supernatants (presented as pg/ml). IFN-γ production was measured using a modification of a previously described ELISA with reagents kindly provided by Dr. Robert Schreiber (Washington University, St. Louis, MO) (31). Briefly, H1.5 (anti-IFN-γ) ascites fluid (3.5 mg/ml) was precoated on 96-well Nunc ImmunoPlate 1 (Nunc, Naperville, IL). Samples were added in serial dilutions at a final volume of 100 μl/well and incubated for 1 h at 37°C. The wells were blocked with 2% BSA for 1 h at room temperature, washed, and incubated with a polyclonal rabbit antiserum (1/10000) for 45 min. Bound rabbit Abs were detected using a 1/3000 dilution of alkaline phosphatase-coupled donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), followed by the phosphatase substrate p-nitrophenyl phosphate disodium (Sigma) at 1 mg/ml. An IFN-γ standard was used to quantify units of activity.

Results

T cells from CTLA-4-deficient mice are autoproliferative, but unresponsive to CD28 costimulation

T cells from CTLA-4KO mice have previously been reported to express activation markers such as IL-2R and CD69 (19, 22). Since CTLA-4 has been shown to regulate several intermediates in the CD28 pathway (12), it was of interest to examine the CD28 responses of T cells from CTLA-4KO mice. In contrast to the previous reports of T cells from CTLA-4-deficient mice being hyperproliferative in response to TCR stimulation, the T cells from CTLA-4-deficient mice in the current study had slightly lower responses than the WT T cells to mitogenic stimuli like anti-CD3 mAb (Fig. 1). T cells from CTLA-4KO mice did have a higher level of spontaneous proliferation ranging from 3–6-fold in different experiments (CTLA-4KO = 15,900 cpm vs CTLA-4WT = 2,800 cpm for the experiment shown in Fig. 1), similar to the previous reports. The differences in the present study versus previous reports most likely are a result of the breeding back to a different background (NOD versus 129) and the fact that T cells were harvested from mice late in disease progression. Furthermore, differences in the source of lymphocytes, potential role of APCs, and the concentration of the anti-CD3 mAbs may all have affected the results. However, the absence of a hyperproliferative response by CTLA-4KO T cells was reproducible (in six of eight experiments), although the finding of reduced proliferation as observed in Fig. 1 was not always observed. Earlier studies have shown that CD28 signal transduction is down-regulated following T cell activation (32). Therefore, the costimulatory effect of anti-CD28 mAb was compared among T cells isolated from CTLA-4KO and WT mice. Whole LN (Fig. 1) or purified (data not shown) T cells from CTLA-4KO or WT mice were stimulated with maximal anti-CD28 over a wide range of anti-CD3 mAb concentrations (10–0.1 μg/ml; data shown for 2 and 0.1 μg/ml). Although costimulation with anti-CD28 mAb resulted in a 3–5-fold increase in the proliferative response of the T cells from the WT littersmates, T cells from the CTLA-4KO mice were unresponsive to the anti-CD28 mAb. The addition of rIL-2 increased proliferation of T cells from both CTLA-4KO and normal mice, suggesting that the deficit was restricted to the CD28 costimulatory pathway and IL-2 production per se. In addition, restimulating the CTLA-4KO T cells overnight did not improve their proliferative responses (data
not shown). Together these results suggest that the hyperactivation of the CTLA-4KO T cells in vivo compromises their proliferative capacity in vitro at least in part due to defective CD28-mediated T cell costimulation.

**T cells from CTLA-4KO mice do not exhibit increased cell death**

One reason the CTLA-4KO T cells exhibited reduced proliferation and defective anti-CD28-mediated costimulation could be related to kinetic differences as compared with the WT T cells. Therefore, the proliferative capacity and viability of CTLA-4 T cells throughout the culture period were assessed. The proliferative responses of WT T cells peaked at 48 h in response to the combination of anti-CD3 and anti-CD28 mAbs. In contrast, the [3H]thymidine incorporation in cells from CTLA-4-deficient mice was higher than normal T cells at 24 h (perhaps related to the high degree of in vivo activation), but did not increase any further with time or with anti-CD28 costimulation (Fig. 2). Thus, the inability of CTLA-4KO T cells to respond to mitogenic stimuli was not due to increased cell death. Together, the results suggest that the reduced response of CTLA-4KO T cells was due to decreased cell expansion, most likely a consequence of the hyperstimulation in vivo. This conclusion is consistent with previous observations that preactivated T cells are refractory to restimulation (33).

**T cells from CTLA-4-deficient mice express higher levels of Bcl-xL**

Although the T cells from CTLA-4-deficient mice proliferated poorly to TCR/CD28 activation, the CTLA-4KO T cells had an increased viability versus WT T cells at the end of the culture period. To assess whether the increased survival of CTLA-4KO T cells relative to WT T cells correlated with the expression of cell survival factors, the level of intracellular Bcl-xL protein was determined (Fig. 3). T cells directly isolated from CTLA-4-deficient mice expressed even higher levels of Bcl-xL as compared with WT T cells (Fig. 3A). Moreover, the higher Bcl-xL expression was evident after 2 days in culture with anti-CD3. Finally, the addition of anti-CD28 to the activation cultures increased Bcl-xL expression in WT T cells, but had no effect in the T cells isolated from the CTLA-4KO mice. These results extend the findings above, demonstrating that the T cells isolated from CTLA-4KO mice are refractory to CD28 signaling. Furthermore, the results suggest that CTLA-4 may control Bcl-xL expression during T cell activation. This is in contrast to the recently reported results, in which inhibitory anti-CTLA-4 mAbs did not have any effect on Bcl-xL expression (34). This result may reflect differences between the...
mAb-induced CTLA-4 regulation and the consequences of CTLA-4 deficiency in vivo.

**Th2 skewing of T cells from CTLA-4KO mice**

Proliferative responsiveness can be influenced by the state of differentiation of the T cells. Therefore, the cytokine profile of CTLA-4-deficient T cells was examined. The cytokine profile of activated T cells from 13–15-day-old, CTLA-4-deficient mice was highly skewed toward the Th2 phenotype. CTLA-4-deficient T cells secreted high levels of IL-4 and IL-5 as compared with control WT T cells (data shown for IL-4 only), whereas the levels of IL-2 and IFN-γ secreted upon anti-CD3 stimulation were comparable with the normal T cells (Fig. 4). It is important to note that these results differ from previous studies (18, 22) most likely due to strain differences. B6/129 mice are prone to Th1-type responses, whereas T cells from mice bred to the NOD background produce strong Th2 responses (Bluestone et al., unpublished observations). In addition, differences in the source of lymphocytes, potential role of APCs, and the concentration of the anti-CD3 mAbs may all have influenced the results. As predicted from the proliferation assays, costimulation of the WT T cells with anti-CD28 mAb significantly increased the IL-2 and IFN-γ secretion, but did not have any effect on cytokine secretion by CTLA-4-deficient cells.

**The CD4+ T cell subset is the source of the Th2 cytokines**

To identify the subset of T cells that was the source of Th2 cytokines in CTLA-4-deficient mice, T cells were separated into CD4+ and CD8+ T cell subsets. A comparison of whole LN cells with purified CD4+ and CD8+ T cells showed that the CD4+ T cells from CTLA-4-deficient mice secreted high levels of IL-4 in response to anti-CD3 or anti-CD3 plus anti-CD28 (Fig. 5). The IL-4 production by the purified CD4+ T cells was higher than observed with whole LN, as the percentage of CD4+ T cells in whole LN cultures was lower. The CD4+ T cells secreted both IL-2 and IFN-γ, although the production of the Th1 cytokines in the CTLA-4-deficient T cell cultures was lower than normal and did not increase upon anti-CD28 costimulation.

The cytokine profile of the CTLA-4-deficient CD8+ T cell subset was different from the CD4+ T cells. First, there was no IL-4 detectable in the culture supernatant of activated CD8+ T cells. Furthermore, the level of IFN-γ production by the CD8+ T cells was higher than was found in the CD4+ T cell subset. Finally, CD28-mediated T cell costimulation was normal in the isolated CD8+ T cell subset. Therefore, the Th2 skewing as well as the CD28 unresponsiveness observed in CTLA-4KO mice was manifested selectively in the CD4+ subset of T cells. In this regard, it is interesting to note that Allison and colleagues have implicated the CD4+, not CD8+, T cell subset in mediating disease in the CTLA-4KO mice (23). Furthermore, Bachmann et al. showed in a TCR transgenic model that CTLA-4KO CD8+ LCMV-specific T cells did not seem to be regulated by CTLA-4 (35).
Treatment with mCTLA-4Ig restores normal T cell phenotype and CD28 responsiveness, and prevents Th2 skewing in CTLA-4-deficient mice

We examined whether blockade of Th2 development would alter the cellular phenotype of the proliferating T cells and prevent disease progression. Previous studies have shown that Th2 development can be blocked using a CD28 antagonist such as mCTLA4Ig. Therefore, CTLA-4-deficient mice were treated with 100 µg/animal mCTLA4Ig. The treatment was begun at day 12 postbirth when there were already signs of an activated T cell phenotype in most animals. Whereas untreated mice died by 3 wk of age, mCTLA4Ig treatment delayed the onset of disease by 2–3 wk and, in some cases, prolonged survival to as much as 45 days (Fig. 6). In fact, the CTLA-4KO mice could be kept alive even longer by continuous mCTLA4Ig treatment or by breeding the CTLA-4KO mice to transgenic mice, producing high serum levels of mCTLA4Ig driven by the K14 promoter (36). Many of these animals stayed alive for greater than 8 mo. Those animals that died succumbed to a lymphoproliferative disease similar to that observed in 2- to 4-wk-old CTLA-4KO mice (data not shown). Thus, there was an ongoing peripheral activation of T cells occurring even in adult mice that could be delayed by increasing the dose or the duration of mCTLA4Ig treatment.

Phenotypic analysis of the mCTLA4Ig-treated mice revealed a significant decrease in T cell activation. As previously reported, the majority of T cells from CTLA-4-deficient mice expressed an activated phenotype (CD44high (Pgp-1), CD69 high, CD62L low (Mel-14)) when examined at 2–3 wk of age, mCTLA4Ig treatment delayed the onset of disease by 2–3 wk and, in some cases, prolonged survival to as much as 45 days (Fig. 6). In fact, the CTLA-4KO mice could be kept alive even longer by continuous mCTLA4Ig treatment or by breeding the CTLA-4KO mice to transgenic mice, producing high serum levels of mCTLA4Ig driven by the K14 promoter (36). Many of these animals stayed alive for greater than 8 mo. Those animals that died succumbed to a lymphoproliferative disease similar to that observed in 2- to 4-wk-old CTLA-4KO mice (data not shown). Thus, there was an ongoing peripheral activation of T cells occurring even in adult mice that could be delayed by increasing the dose or the duration of mCTLA4Ig treatment.

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Since T cells from mCTLA4Ig-treated mice had a normal phenotype, the proliferative and cytokine responses of these T cells were examined. LN cells from WT, mCTLA4Ig-treated CTLA-4KO mice demonstrated proliferative responses to anti-CD3 mAb comparable with WT T cells over the full time course (Fig. 8). Furthermore, the unresponsiveness to anti-CD28 mAb observed with CTLA-4KO T cells was not evident in T cells from mCTLA4Ig-treated CTLA-4KO mice. These T cells responded normally to anti-CD28 mAb and IL-2 costimulation.

Finally, the cytokine profile of the T cells from mCTLA4Ig-treated CTLA-4-deficient mice was examined. T cells from treated
mice produced cytokines qualitatively and quantitatively similar to those of normal mice (Fig. 9). T cells from mCTLA4Ig-treated CTLA-4-deficient mice secreted IL-2 and IFN-γ upon stimulation with anti-CD3 mAb, and the levels increased upon costimulation with anti-CD28. Interestingly, the levels of IFN-γ in the mCTLA4Ig-treated CTLA-4KO mice were significantly higher than controls. Similarly, only a small amount of IL-4 was produced by mCTLA4Ig-treated CTLA-4KO T cells in response to anti-CD3 plus anti-CD28 mAbs. These results are consistent with the possibility that a subset of differentiated T cells remained in these animals that were not affected by the mCTLA4Ig therapy or that a small number of activated T cells appeared after the mCTLA4Ig treatment was discontinued.

Discussion

Several in vivo and in vitro studies support a critical role for the CTLA-4 pathway in peripheral tolerance. Most significantly, the absence of CTLA-4 expression leads to a profound autodestruction and the premature death of CTLA-4-deficient mice. In the present study, the proliferation and cytokine profile of T cells isolated from these animals were examined. Although T cells from CTLA-4-deficient mice underwent significant activation in vivo, the T cells were not hyperproliferative in response to TCR stimulation in vitro. In fact, in many experiments, the CTLA-4KO T cells showed diminished responses. These results were reproducible and significant, but differ from previous reports. The difference in the present study most likely reflects differences in the genetic background of the mice and the strong skewing toward the less reactive Th2 subset. A time course analysis of CTLA-4KO T cells showed that these hyperactive cells were viable as late as day 4 to 5 in culture, indicating that they were not as susceptible to activation-induced cell death as WT T cells. Since CD28 is known to regulate the expression of survival factors such as Bcl-xL, it was of interest to compare the levels of Bcl-xL in T cells from CTLA-4KO mice. T cells from CTLA-4-deficient mice expressed higher levels of Bcl-xL on day 0 as compared with WT T cells. Stimulation of the CTLA-4KO with anti-CD3 mAb led to further up-regulation of Bcl-xL similar to that observed in WT T cells. It has been reported that CD28 costimulation induces the expression of survival factors such as Bcl-xL (7), leading to resistance to activation-induced cell death. These results are consistent with the possibility that a subset of differentiated T cells remained in these animals that were not affected by the mCTLA4Ig therapy or that a small number of activated T cells appeared after the mCTLA4Ig treatment was discontinued.

FIGURE 7. mCTLA4Ig treatment prevents T cell activation in CTLA-4-deficient mice. LN cells from 14-day-old CTLA-4-deficient, WT or 4- to 5-wk-old mCTLA4Ig-treated CTLA-4-deficient mice were stained for expression of CD44 (Pgp-1), CD62L (Mel-14), and CD69 on T cells, as described in Materials and Methods. Data were collected for 5 × 10^5 live cells using a FACScan and are presented as histogram plots of CD44, CD62L, or CD69 expression on CD4^+/CD8^- cells.

FIGURE 8. mCTLA4Ig treatment restores CD28-mediated costimulation of T cells from CTLA-4-deficient mice. LN cells from 4- to 5-wk-old WT, mCTLA4Ig-treated WT, and CTLA-4-deficient mice were stimulated at 2 × 10^5 cells/well with anti-CD3 e mAb (0.1 mg/ml) with or without anti-CD28 mAb (1 mg/ml), or IL-2 (25 U/ml) for different times. The proliferation was determined based on [3H]thymidine uptake for the final 8 h of culture. Data are expressed as the mean of triplicate cultures.
This is in contrast to the recent results in which CTLA-4 mAb suggests that CTLA-4 may be involved in down-regulation of Bcl-xL expression of Bcl-xL in T cells from CTLA-4-deficient mice that inhibited proliferation had no effect on Bcl-xL expression (34). However, the effects of hyperactivation in vivo may be different from in vitro, and the genetic absence in vivo of CTLA-4 may result in differences not observed following cross-linking of CTLA-4 by mAbs in vitro. It should be noted that there is no direct evidence to show that the T cells expressing higher levels of bcl-xL are the cells that survive longer in culture, although this is not an unreasonable conclusion.

A productive immune response requires activation of T cells via the TCR and the CD28 costimulatory pathway. Our current studies indicate that blocking the CD28/B7 interaction with mCTLA4Ig either prevents the activation of T cells or induces anergy in T cells. In an attempt to differentiate between the two possibilities, T cells from mCTLA4Ig-treated mice were compared with WT T cells in phenotype and in functional assays. The T cells from mCTLA4Ig-treated mice did not express any activation markers, and had similar proliferation and cytokine profile as the WT T cells. These results indicated that mCTLA4Ig treatment of the WT T cells. These results indicated that mCTLA4Ig treatment of the WT T cells prevented the activation of T cells or inducing anergy in T cells. However, in multiple experiments, the T cells from mCTLA4Ig-treated mice consistently produced higher levels of IFN-γ than normal WT T cells, suggesting that some level of residual activation existed in these mice, although these cells did not mediate lethal disease. In this regard, a comparison of cytokine profiles of the T cells from the CTLA-4-deficient mice showed that the CD4+ T cells produced much higher amounts of IL-4 and IL-5, cytokines characteristic of a Th2 phenotype. We have reported previously that a strong CD28 signal or blocking the CTLA-4 signal skew the T cells toward a Th2 phenotype. Thus, the presence of Th2 type of T cells in CTLA-4-deficient mice may be due to a strong or repeated CD28 signal without down-regulation by CTLA-4 (41–43). Alternatively, the skewing of the T cell phenotype could be due to preferential effects on Th2 cell proliferation and/or survival in vivo.

Finally, the present studies support a role for CTLA-4 in regulating the Th1 versus Th2 balance, and suggest that the Th2-type T cells may play a critical role in the pathogenesis of this disease in down-regulating the immune response possibly through the regulation of survival factors.

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