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Lymphoproliferative Disorder in CTLA-4 Knockout Mice Is Characterized by CD28-Regulated Activation of Th2 Responses¹

Roli Khattri,^{2*} Julie A. Auger,^{2*} Matthew D. Griffin,* Arlene H. Sharpe,^{3†} and Jeffrey A. Bluestone^{3,4*}

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-x_L, 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.

T cell activation depends on TCR-independent regulatory cell-cell interactions that control T cell signaling. Among the critical cell surface molecules that regulate T cell activation are CTLA-4 and CD28, both of which bind the same ligands, B7-1 (CD80) and B7-2 (CD86) (1–3). However, the two cell surface glycoproteins have antagonistic functions. CD28 engagement enhances T cell responses and T cell survival by enhancing IL-2 mRNA transcription and stability (4, 5), high affinity IL-2R expression, and the induction of cell survival factors such as Bcl-x_L (6–8). In contrast, CTLA-4 down-regulates T cell activation (9–11).

Evidence for an inhibitory role of CTLA-4 has come from both in vitro as well as in vivo studies. In vitro experiments have shown that cross-linking of CTLA-4 inhibits intracellular signaling, proliferation, and IL-2 secretion, while blocking CTLA-4/B7 engagement leads to augmented T cell responses (9–14). The immune enhancing effects of CTLA-4 blockade have been demonstrated in vivo as well, wherein blocking CTLA-4 augments Ag and super-

antigen-induced expansion of T cells (15) and exacerbates a number of autoimmune diseases (16, 17). Most importantly, an analysis of the CTLA-4 knockout (KO) mice supported a critical role for CTLA-4 in peripheral tolerance (18, 19). The CTLA-4-deficient mice exhibit a massive polyclonal expansion of T cells and multiorgan tissue destruction, and die at an age of 2–3 wk. Most T cells in these animals express an activated phenotype (CD69⁺, CD62L^{low}, CD44^{high}) and proliferate spontaneously in vitro. Current evidence suggests that the accumulation of the activated T cell population is not due to a defect in thymic selection (20, 21). Instead this accumulation is due to uncontrolled activation and expansion of peripheral T cells, perhaps as a consequence of altered control of normal homeostasis (22). Finally, the induction of autoimmune disease in CTLA-4-deficient mice is CD28/B7 dependent and mediated by the CD4⁺ subset of T cells, since treatment of these mice with murine CTLA4Ig (mCTLA4Ig)⁵ or depletion of the CD4⁺ subset of T cells blocks the lymphoproliferative disease (23).

The current study was designed to define the characteristics of the T cell population mediating the autoimmune disease. Similar to previous reports, we observed that the T cells present at the time of onset of lymphoproliferative disease in CTLA-4-deficient mice expressed an activated phenotype and a significant level of auto-proliferation. However, the T cells were defective in CD28 costimulation and demonstrated a predominantly Th2 phenotype. Although the T cells from CTLA-4-deficient mice demonstrated a blunted proliferative ability, the T cells survived longer in culture correlating with higher Bcl-x_L expression most likely due to the chronic stimulation of these cells. Furthermore, we observed that the lymphoproliferative disease in CTLA-4-deficient mice was prevented by treatment with mCTLA4Ig even when started as late

*Committee on Immunology, Ben May Institute for Cancer Research, Department of Pathology, University of Chicago, Chicago, IL 60637; and [†]Immunology Research Division, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

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² R.K. and J.A.A. have contributed equally to the study and should be considered co-first authors.

³ A.H.S. and J.A.B. are co-senior authors on this study.

⁴ Address correspondence and reprint requests to Dr. J. A. Bluestone, Ben May Institute for Cancer Research, University of Chicago, MC1089, 5841 South Maryland Avenue, Chicago, IL 60637. E-mail address: jbluest@immunology.uchicago.edu

⁵ 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.

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'-ATGGTGTGGCTAGCAGCCATG and 3'-TTGGATGGT-GAGGTTCACTC) and the neomycin resistance gene (5'-ATTGAACAA-GATGGATTGCAC and 3'-CGTCCAGATCATCTGATC). 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 5×10^5 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% Na₂S₂O₃) for 30 min at 4°C. The cells were washed twice with PBS-A buffer and, if necessary, counterstained with PE-coupled streptavidin (Southern Biotechnology, Birmingham, AL) for 15 min at 4°C, and washed once with PBS-A buffer before analysis. Stained cells (1×10^4) were analyzed on a FAC-Scan (Becton Dickinson, Mountain View, CA) using LYSIS II and WinMDI software packages (WinMDI software by Joseph Trotter; available from flosun.salk.edu/in/pub/pc). 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-2Rα (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 stimulated 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 before analysis on a FACScan. All samples were run for a fixed time period (15 s). Cells staining PI^{bright} were considered to be the dead cells, and PI^{negative} cells were considered to be alive.

Bcl-x_L staining

Aliquots of 1×10^6 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_L 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⁺ cells were depleted further using a mixture of anti-heat stable Ag (J11d) (25) and anti-I-A^b (25-9-3) (26) culture supernatants plus rabbit complement (Pel-Freez, Brown Deer, WI). To enrich for CD4⁺ T cells, the CD8⁺ T cells were depleted with anti-CD8 mAb (3.155) (27); to enrich for CD8⁺ cells, the CD4⁺ 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 cell subsets used in these assay were >95% pure. T cells (2×10^5) were stimulated with serial dilutions of anti-CD3ε (145-2C11) (29) with or without anti-CD28 (PV-1, 1 μg/ml) (30) and/or IL-2 (25 U/ml). In experiments utilizing separated T cell subsets, 2×10^5 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 [³H]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 (1/1000) 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 autoproductive, 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 littermates, 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, resting the CTLA-4KO T cells overnight did not improve their proliferative responses (data

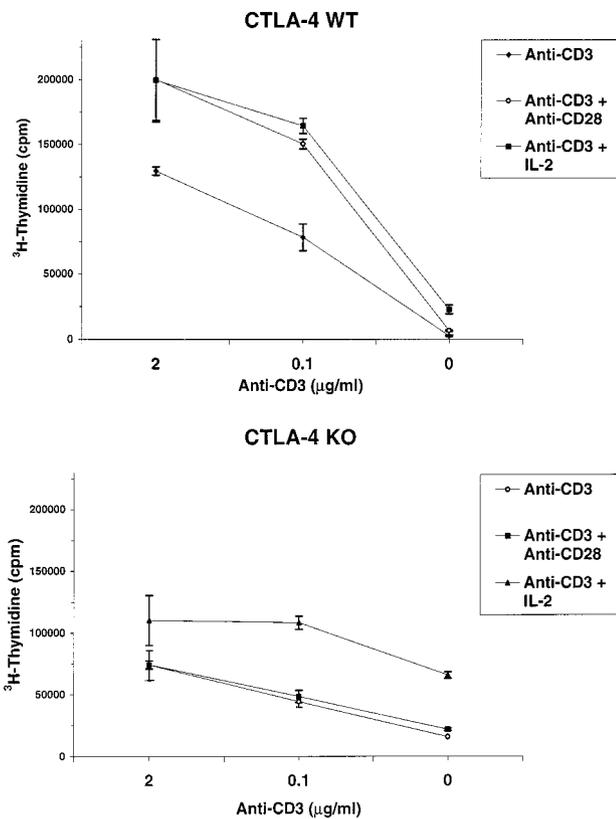


FIGURE 1. T cells from CTLA-4KO mice are unresponsive to CD28 costimulation. LN cells from WT and CTLA-4-deficient mice were stimulated at 2×10^5 cells/well with anti-CD3 ϵ mAb at 2 or 0.1 $\mu\text{g/ml}$ with or without anti-CD28 mAb (1 $\mu\text{g/ml}$) or IL-2 (25 U/ml). Cells were pulsed at 48 h with [³H]thymidine and harvested 8 h later. Data are expressed as the mean of triplicate cultures.

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 difference in proliferative capacity of the CTLA-4KO T cells was not due to a different kinetics of the immune response. Alternatively, it was possible that the CTLA-4KO T cells underwent more rapid apoptosis following T cell stimulation. Direct examination of cell death (the ratio of dead/live cells during the proliferative assay) revealed that CTLA-4KO cells were, if anything, more resistant to activation-induced apoptosis. The WT T cells had slightly better viability than the CTLA-4KO T cells at the early time points of the assay (WT at

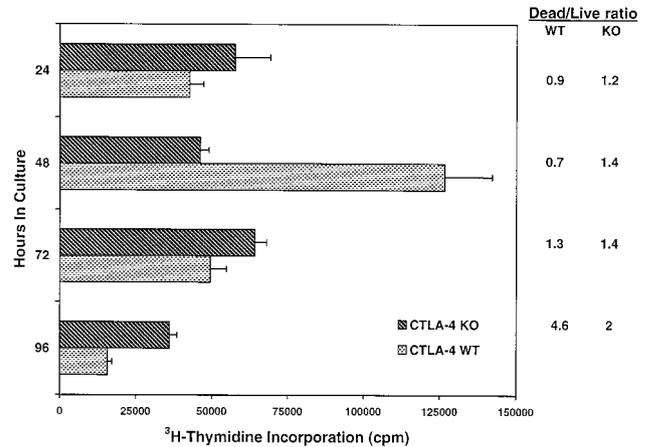


FIGURE 2. CTLA-4-deficient T cells are refractory, but survive longer in culture. LN T cells from WT or CTLA-4-deficient mice were stimulated at 2×10^5 cells/well with anti-CD3 ϵ (0.1 $\mu\text{g/ml}$) plus anti-CD28 (1 $\mu\text{g/ml}$) for different times. The proliferation was determined based on [³H]thymidine uptake for the final 8 h of culture. Data are expressed as the mean of triplicate cultures. To measure the dead versus the live cell count of the cultures, cells were harvested from three wells of a 96-well microtiter plate and pooled. The cells were stained with 10 μl of 0.1 mg/ml PI (without any washes) just before analysis and were run for a fixed amount of time (15 s). The data are presented as a ratio of the dead/live cells.

24 h = 0.9 and 48 h = 0.7; CTLA-4KO at 24 h = 1.2 and 48 h = 1.4). However, the viability of the CTLA-4KO T cells was greater than the WT T cells at the late time points. For instance, the T cells from the CTLA-4KO mice exhibited a dead/live ratio of 2 as compared with 4.6 for the normal T cells. We have shown previously that PI staining and subsequent determination of the dead/live cell ratio is a very sensitive tool to examine apoptosis in the context of cell expansion (8). 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-x_L

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-x_L protein was determined (Fig. 3). T cells directly isolated from CTLA-4-deficient mice expressed even higher levels of Bcl-x_L as compared with WT T cells (Fig. 3A). Moreover, the higher Bcl-x_L expression was evident after 2 days in culture with anti-CD3. Finally, the addition of anti-CD28 to the activation cultures increased Bcl-x_L 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-x_L 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-x_L expression (34). This result may reflect differences between the

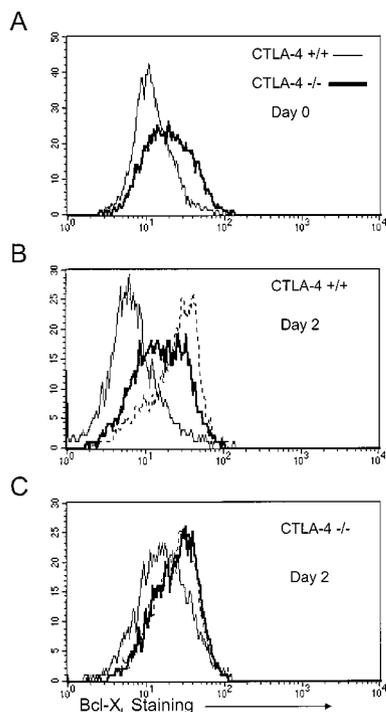


FIGURE 3. CTLA-4-deficient T cells express higher levels of Bcl- x_L . LN T cells from 14-day-old mice were stained with anti-Bcl- x_L mAb, as described in *Materials and Methods*. An isotype-matched control was used to determine background staining. Cells were analyzed on a FACScan. *A*, Bcl- x_L expression in T cells directly out of the animal. Thin line, CTLA4^{+/+}; thick line, CTLA4^{-/-}. *B* and *C*, Bcl- x_L expression at 48 h after stimulation. Thin line, no stimulation; thick line, anti-CD3 mAb (1 μ g/ml); dashed line, anti-CD3 mAb (1 μ g/ml) plus anti-CD28 (1 μ g/ml).

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

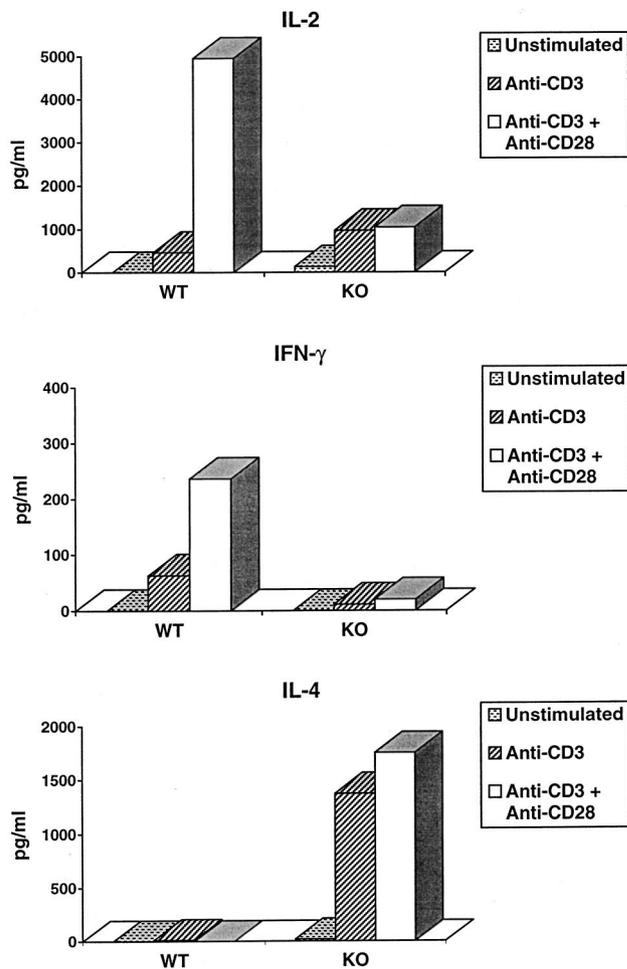


FIGURE 4. Th2 skewing of cytokine responses of T cells from CTLA-4-deficient mice. Purified LN T cells from WT and CTLA-4-deficient mice were stimulated at 2×10^5 cells/well with anti-CD3 ϵ mAb (0.1 μ g/ml) or anti-CD3 ϵ mAb plus anti-CD28 (1 μ g/ml). Cytokine production by T cells was measured in supernatants harvested 24 h after stimulation for IL-2 and 48 h later for IFN- γ and IL-4. Cytokine levels were determined by ELISA.

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).

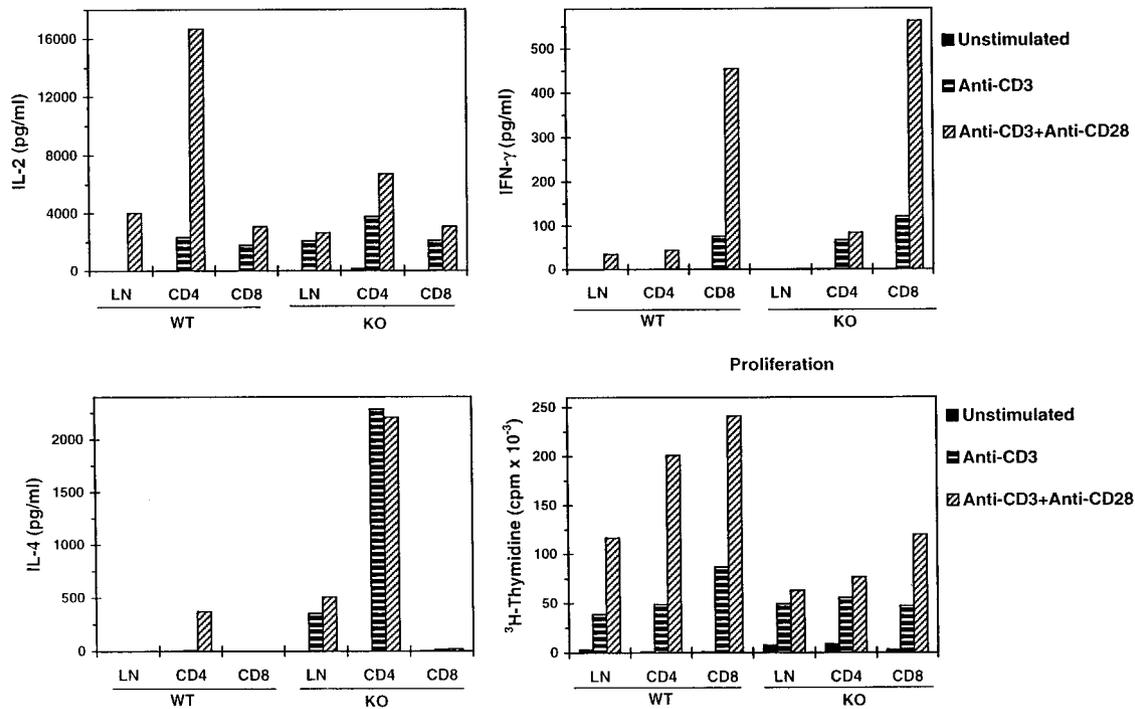


FIGURE 5. Th2 skewing in CTLA-4-deficient mice is restricted to the CD4⁺ subset of T cells. Whole LN T cells or purified CD4⁺ and CD8⁺ T cells were stimulated with 2×10^5 cells/well with anti-CD3 ϵ mAb (0.1 μ g/ml) or anti-CD3 ϵ mAb plus anti-CD28 (1 μ g/ml). Cytokine production by T cells was measured in supernatants harvested 24 h after stimulation for IL-2 and 48 h later for IFN- γ and IL-4 concentrations by ELISA. To measure proliferation, cells were pulsed at 48 h with [³H]thymidine and harvested 8 h later. Data are expressed as the mean of triplicate cultures.

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 μ g/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 (CD44^{high} (Pgp-1), CD69^{high}, CD62L^{low} (Mel-14)) when examined at 2–3 wk of age (50–75% Mel-14^{low}) (Fig. 7). The activation status of LN T cells of 3-wk-old mCTLA4Ig-treated mice appeared normal. The majority of T cells did not express CD69 and were CD62L^{high} (Fig. 7). Interestingly, the number of T cells isolated from the lymphoid tissues of these animals was comparable with normal animals, suggesting that the

mCTLA4Ig prevented both clonal expansion and T cell differentiation.

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

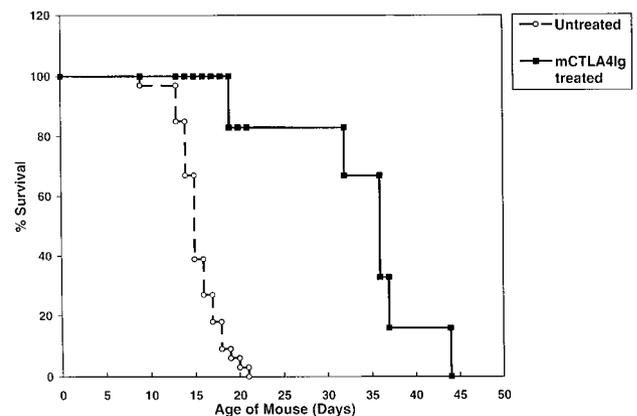
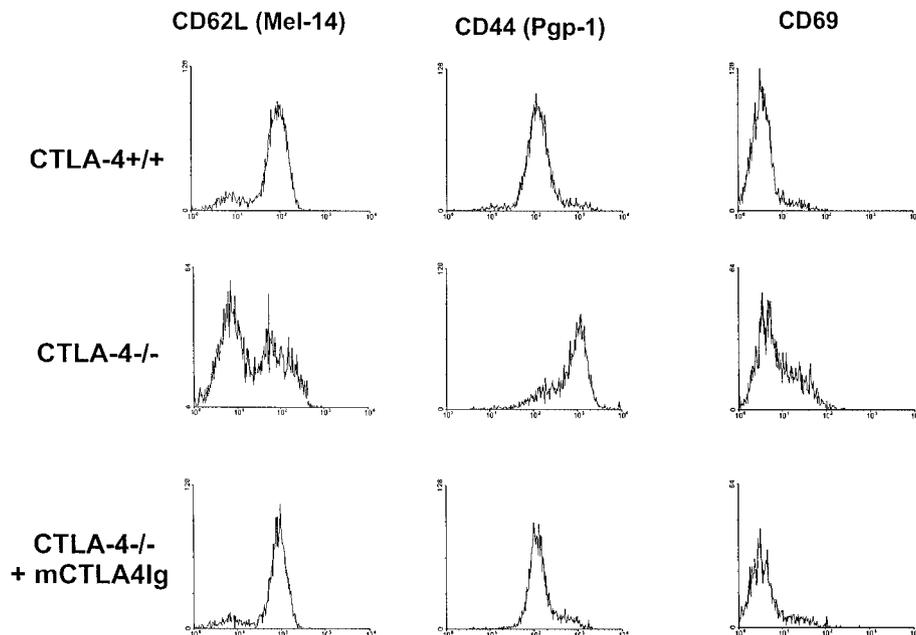


FIGURE 6. mCTLA4Ig treatment delays death in CTLA-4-deficient mice. CTLA-4-deficient mice were treated with 100 μ g of mCTLA4Ig ($n = 10$) given i.p. on days 12, 15, and 18 after birth or left untreated ($n = 17$).

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 5×10^3 live cells using a FACScan and are presented as histogram plots of CD44, CD62L, or CD69 expression on CD4⁺/CD8⁺ cells.



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-x_L, it was of interest to compare the levels of Bcl-x_L in T cells from CTLA-4KO mice. T cells from CTLA-4-deficient mice expressed higher levels of Bcl-x_L 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-x_L similar to that observed in WT T cells. It has been reported that CD28 costimulation induces the expression of survival factors such as Bcl-x_L (7), leading to resistance to activation-induced cell

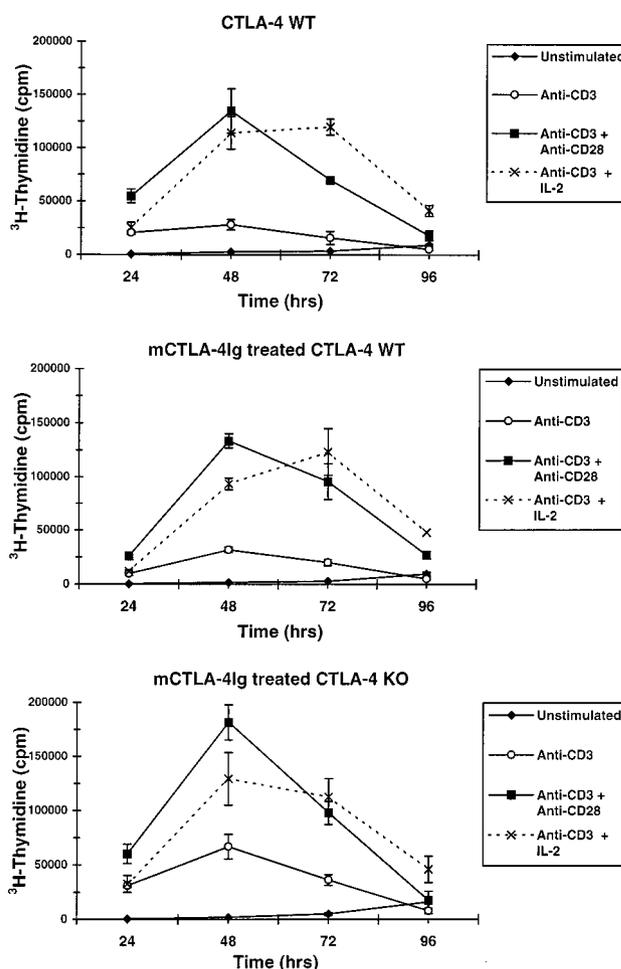


FIGURE 8. mCTLA4Ig treatment restores CD28-mediated costimulation of T cells from CTLA-4-deficient mice. LN cells from 4- to 5-wk-old WT, mCTLA4Ig-treated WT, and CTLA-4-deficient mice were stimulated at 2×10^5 cells/well with anti-CD3 ϵ mAb (0.1 μ g/ml) with or without anti-CD28 mAb (1 μ g/ml), or IL-2 (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.

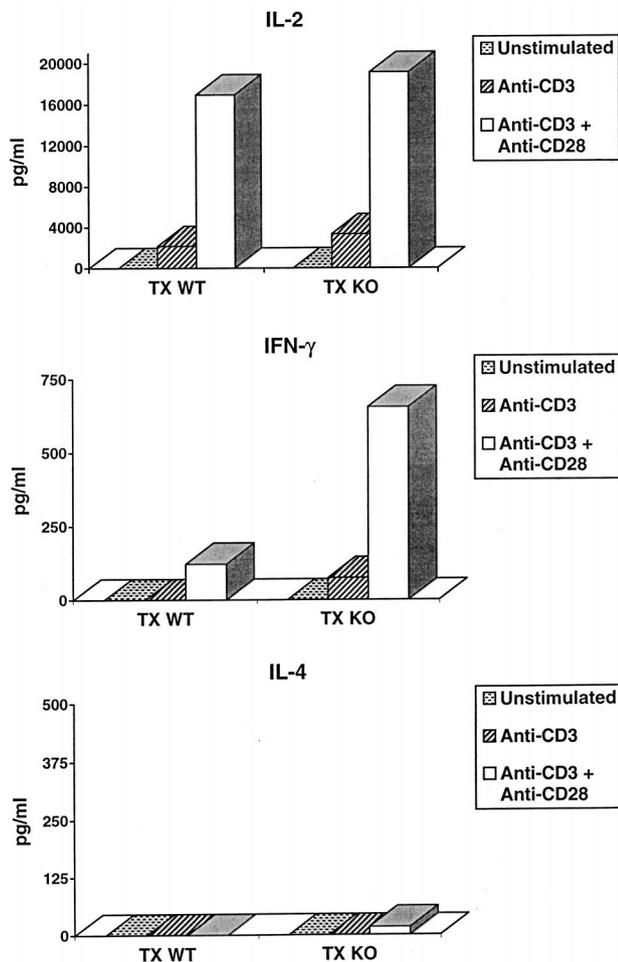


FIGURE 9. Th2 skewing of cytokine responses of T cells from CTLA-4-deficient mice is prevented by mCTLA4Ig treatment. Purified LN T cells from mCTLA4Ig-treated (TX) WT and CTLA-4-deficient (KO) mice were stimulated at 2×10^5 cells/well with anti-CD3 ϵ (0.1 μ g/ml) or anti-CD3 ϵ plus anti-CD28 (1 μ g/ml). Cytokine production by T cells was measured in supernatants harvested 24 h after stimulation for IL-2 and 48 h later for IFN- γ and IL-4. Cytokine levels were determined by ELISA.

death early in culture (37); however, the CTLA-4KO T cells did not respond to CD28 costimulation, suggesting that either TCR or other costimulatory signals regulated Bcl-x_L expression. Constitutive expression of Bcl-x_L in T cells from CTLA-4-deficient mice suggests that CTLA-4 may be involved in down-regulation of Bcl-x_L. This is in contrast to the recent results in which CTLA-4 mAb that inhibited proliferation had no effect on Bcl-x_L expression (34). However, the effects of hyperactivation *in vivo* may be quite 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-x_L 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 as late as day 12 after birth delayed the onset of disease. These results are an extension of recent reports in which the treatment of CTLA-4-deficient mice with mCTLA4Ig from the day of birth delayed disease onset. The basis for the CD28-dependent T cell activation is unclear, but may represent the activation of T cells by low af-

finity self Ags or environmental pathogens. This is consistent with previous studies showing that low affinity responses are exquisitely CD28 dependent, whereas high affinity Ags can activate T cells in a CD28-independent manner (38, 39). Alternatively, recent studies by several groups have shown that survival of peripheral T cells depends on sustained interactions of the TCR with self MHC Ag (40). To date, the role of CD28 in long-term T cell survival has not been examined. However, it remains possible that CTLA-4 normally controls this limited self-reactivity. In either case, the uncontrolled hyperreactivity of CTLA-4KO T cells is CD28 dependent *in vivo*, although the hyperactive T cells from the CTLA-4KO mice did not respond to CD28 costimulation *in vitro*. These results suggest that once the CTLA-4KO T cells are hyperactivated, CD28 costimulation is less apparent. It has been reported that CTLA-4 engagement prevents extracellular signal-regulated kinase (ERK) and Jun NH2-terminal kinase (JNK) activation (12). It is possible that signals through CTLA-4 are required for switching off these kinases, and in the absence of CTLA-4 down-regulation, the T cells are rendered refractory to further CD28 signaling mediated via these kinases.

CTLA-4 does not seem to play a critical role in thymic development. The CTLA-4-deficient mice have normal numbers of T cell subsets that function normally *in vitro* (20, 21). However, it remains possible that subtle changes in TCR repertoire and affinity may lead to a lower threshold for activation in the periphery. In either case, the treatment of CTLA-4KO mice with mCTLA4Ig as late as day 12 postbirth can reverse disease progression, suggesting that CD28 and CTLA-4 play an ongoing role in the regulation of peripheral T cell activation. Thus, blocking 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 was indeed preventing T cell activation and not inducing anergy. However, in multiple experiments, the T cells from mCTLA4Ig-treated CTLA4KO 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 skews 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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