Brief Definitive Report

B7-1 or B7-2 Is Required to Produce the Lymphoproliferative Phenotype in Mice Lacking Cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4)

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Summary

The costimulatory molecules B7-1 and B7-2 regulate T lymphocyte activation by delivering activating signals through CD28 and inhibitory signals through cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). The importance of CTLA-4-mediated inhibition was demonstrated by the uncontrolled T cell activation and lymphoproliferative disease that develops in CTLA-4-deficient (−/−) mice. To examine the role of B7 signaling in the activation of CTLA-4-deficient T cells, we bred CTLA-4−/− mice with mice lacking B7-1, B7-2, or both B7 molecules. The CTLA-4−/−B7-1−/− and the CTLA-4−/−B7-2−/− mice develop lymphoproliferation and enhanced T cell activation. Mice lacking CTLA-4, B7-1, and B7-2 have a normal life-span, and do not have lymphocytic infiltrates in any organs, or increased T cell activation. Therefore, the two B7 molecules have overlapping functions, since either B7-1 or B7-2 alone can cause the CTLA-4−/− phenotype. Elimination of both B7-1 and B7-2 from the CTLA-4-deficient mouse abrogates the lymphocyte activation and disease, and does not reveal evidence for additional stimulatory CD28 ligands. The CTLA-4−/− phenotype can be reproduced with anti-CD28 antibody in mice lacking CTLA-4, B7-1, and B7-2, but wild-type mice are unaffected by the same treatment. This suggests that the inhibitory function of CTLA-4 can overcome strong CD28-mediated signaling in vivo.

Key words: cytotoxic T lymphocyte-associated antigen 4 • B7 • knockout mouse • costimulation • T lymphocyte
Here we have used the CTLA-4−/− mouse as a tool to investigate interactions between CD28 and its ligands. We have generated and characterized three novel mouse strains, which are CTLA-4-deficient and also lack B7-1, B7-2, or both B7 molecules. If additional stimulatory CD28 ligands existed, then mice lacking B7-1 and B7-2 would be expected to develop increased T cell activation. Alternatively, if there is not increased T cell activation in these mice, then this would suggest that in the absence of the two known B7 molecules, CD28 is no longer engaged.

We found that mice lacking CTLA-4 and either B7-1 or B7-2 develop the lymphoproliferative phenotype observed in the CTLA-4−/− strain, but that mice deficient in CTLA-4 and B7-1 have a shorter life-span and show greater T cell activation in vivo than the CTLA-4/B7-2−/− deficient mice. In contrast, mice lacking CTLA-4, B7-1, and B7-2 ("triple knockout," CTLA-4/B7-1/B7-2−/− TKO) show no evidence of T cell activation or lymphoproliferation, suggesting that there are no additional stimulatory ligands for CD28. We also show that administration of anti-CD28 antibody to CTLA-4/B7-1/B7-2−/− TKO mice can reproduce the phenotype of the CTLA-4−/− mouse, demonstrating the critical role of CD28 in activating CTLA-4−/− T cells.

Materials and Methods

Mice. Mouse strains lacking CTLA-4 (6), B7-1 (10), B7-2, or both B7 molecules (11) have been described previously. Because CTLA-4-deficient mice die before reaching sexual maturity, the three CTLA-4/B7-1−/− mice were generated by interbreeding CTLA-4 heterozygotes with B7-1−/−, B7-2−/−, or B7-1/B7-2−/− deficient mice. Mice heterozygous for both CTLA-4 and the B7 molecules (11) have been described previously. Because mice can reproduce the phenotype of the CTLA-4−/− mouse, demonstrating the critical role of CD28 in activating CD28, provided by Dr. Vijay K. Kuchroo (Brigham and Women's Hospital) and Harvard Medical School are accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and mice were cared for in accordance with institutional guidelines in a pathogen-free animal facility.

In Vivo Administration of Anti-CD28. Wild-type and CTLA-4/B7-1−/− or B7-2−/− mice received either PV-1 mAb to CTLA-4 or anti-CD28 antibody to CTLA-4/B7-1/B7-2−/− TKO mice. The CTLA-4/B7-1−/− TKO strain survives to adulthood, and was maintained by interbreeding these mice. All strains were genotyped by Southern blots, as described previously for CTLA-4 (6), B7-1 (10), and B7-2 (11). Genotypes were confirmed by flow cytometric analysis of splenocytes stimulated with LPS 20 μg/ml and dextran sulfate 10 μg/ml for expression of B7 molecules, and splenocytes stained with anti-CD3 (mAb 145-2C11) for expression of CTLA-4. All mice were generated on an inbred 129/SvJae background. Brigham and Women's Hospital and Harvard Medical School are accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and mice were cared for in accordance with institutional guidelines in a pathogen-free animal facility.

Cell Preparation and Cultures. Single cell suspensions from spleen and lymph nodes were prepared by dissociating tissue with sterile glass slides. Red blood cells were lysed by incubation in Tris-ammonium chloride for 5 min at 37°C. To assay proliferation, 2 × 10^5 cells/well were cultured in flat-bottomed 96-well plates in media as described previously (6). Cells were pulsed with 1 μCi [3H]thymidine for the last 8 h of the indicated day. Anti-CD3 stimulation used high titer supernatant prepared from the 145-2C11 hybridoma, obtained from the American Type Culture Collection.

Histology. Tissue for light microscopy was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard techniques.

Results

B7-1- or B7-2-mediated Signals Are Required to Produce Lymphoproliferation and Fatal Disease in the CTLA-4−/− Mice. To assess the role of B7-1 and B7-2 in producing lymphoproliferative disease in mice lacking CTLA-4, we crossed the CTLA-4-deficient strain with mice lacking B7-1, B7-2, or both B7 molecules. Mice lacking CTLA-4, B7-1/B7-2−/−, and B7-2 (CTLA-4/B7-1−/−; B7-2−/−) develop the characteristic fatal phenotype of CTLA-4−/− mice. The survival of the CTLA-4/B7-1−/− mice (mean = 21.9 d; n = 8) was shorter than that of the CTLA-4/B7-2−/− mice (mean = 30.5 d; n = 8) (P = 0.02 by Wilcoxon signed rank test). The difference in survival between the CTLA-4/B7-1−/− and the CTLA-4−/− (mean = 17.5 d; n = 11) strains did not reach statistical significance (P > 0.05; Fig. 1). In contrast, CTLA-4/B7-1−/−; B7-2−/− TKO mice consistently live longer than 300 d.

![Figure 1](image-url)

**Figure 1.** Either B7-1- or B7-2-mediated signaling is sufficient to produce fatal lymphoproliferative disease in mice lacking CTLA-4. Mice were observed daily, and survival was recorded. Survival of CTLA-4/B7-1−/−; B7-2−/− TKO mice consistently live longer than 300 d.
B7-1, and B7-2 remain healthy and have a life-span and reproductive capacity comparable to wild-type mice.

Both the CTLA-4/B7-1−/− and CTLA-4/B7-2−/− strains develop splenomegaly, lymphadenopathy, and multiorgan lymphocytic infiltrates with tissue damage, similar to the CTLA-4−/− mice. The CTLA-4/B7-1−/− mice typically develop these pathologic changes earlier than the CTLA-4/B7-2−/− mice. In contrast, CTLA-4/B7-1/B7-2−/− TKO mice develop neither splenomegaly nor lymphadenopathy. Detailed histologic examination of CTLA-4/B7-1/B7-2−/− TKO mice ranging in age from 2 to 8 mo reveals the complete absence of lymphocytic infiltrates (Fig. 2). These results indicate that either B7-1 or B7-2 can provide the necessary activating signal for the lymphoproliferative phenotype in CTLA-4−/− mice, but that the absence of both B7 molecules abrogates the phenotype.

In the absence of CTLA-4, extensive T cell activation occurs only in the presence of B7-1 or B7-2. To assess the degree of T cell activation in these mouse strains in vivo, freshly isolated splenocytes were stained for activation markers and analyzed by flow cytometry. T cells from CTLA-4−/−, CTLA-4/B7-1−/−, and CTLA-4/B7-2−/− mice show significant increases in the percentage that are positive for the activation marker CD69 (Fig. 3) and CD62L-low (data not shown) compared with wild-type mice. In four animals of each strain analyzed at 2 wk, there was a mean of 51% CD69+ T cells in CTLA-4−/−, 48% in CTLA-4/B7-1−/−, and 44% in CTLA-4/B7-2−/− mice. The same trend was observed when staining for CD62L, but the differences did not reach statistical significance. In contrast, <10% of CTLA-4/B7-1/B7-2−/− TKO cells are activated, as assessed by staining for CD69 (Fig. 3) and CD62L (data not shown). None of the CTLA-4/B7-1/B7-2−/− TKO mice analyzed show significant T cell activation. Both the CTLA-4/B7-1/B7-2−/− TKO and B7-1/B7-2−/− strains have consistently fewer activated T cells than wild-type mice.

We have previously reported that unfractionated splenocytes from CTLA-4−/− mice proliferate spontaneously in vitro in the absence of exogenous stimulation (7). This assay serves as another measure of unopposed T cell activation in CTLA-4−/− mice. Proliferation in both CTLA-4/B7-1−/− and CTLA-4/B7-2−/− strains was significantly higher than in wild-type mice (Fig. 4 A). As might be expected from other measures of T cell activation in the two strains, CTLA-4/B7-1−/− cells proliferate more than those from age-matched CTLA-4/B7-2−/− cells. In contrast, CTLA-4/B7-1/B7-2−/− TKO splenocytes do not proliferate spontaneously, suggesting that no CD28 signaling occurs in the absence of B7-1 and B7-2.

Figure 2. Removal of B7-1- and B7-2-mediated signaling prevents the lymphocytic infiltration and necrosis of organs observed in CTLA-4−/− mice. Heart (A, B, and C) and pancreas (D, E, and F) shown (original magnification: ×50). CTLA-4−/− mice (B and E), as well as CTLA-4/B7-1−/− and CTLA-4/B7-2−/− mice (not shown), killed at 2 wk of age show severe pancreatitis and myocarditis, in contrast to wild-type littermates (A and D). CTLA-4/B7-1/B7-2−/− TKO mice killed at 2 mo show no evidence of inflammatory infiltrates (C and F).
We also assessed the in vitro proliferative response of CTLA-4/B7-1/B7-2 TKO T cells to anti-CD3 antibody stimulation. The response of CTLA-4/B7-1/B7-2 TKO splenocytes is markedly reduced compared with wild-type mice (Fig. 4 B). This deficit is indistinguishable from that observed with the B7-1/B7-2 TKO mice. Splenocytes from CTLA-4/B7-1/B7-2 TKO mice which are already activated in vivo showed a proliferative response to anti-CD3 stimulation that was highly variable between experiments, presumably because of varying numbers of in vivo-activated T cells, which undergo activation-induced cell death upon restimulation in vitro.

In Vivo Administration of Anti-CD28 to CTLA-4/B7-1/B7-2 TKO Mice Activates T Cells and Produces Fatal Lymphoproliferative Disease. The results showing that T cell activation in CTLA-4 TKO mice requires B7-1 or B7-2 suggest that B7–CD28 interactions are critical for such activation. If CD28 signaling is sufficient to cause T cell activation in the absence of CTLA-4, then ligating CD28 artificially using anti-CD28 antibody should induce disease in CTLA-4/B7-1/B7-2 TKO mice. To test this, we administered anti-CD28 mAb (PV-1) to adult mice for 2 wk. Mice were analyzed 14–19 d after initial treatment with anti-CD28 because they exhibited features typical of the CTLA-4 lymphoproliferative disease. At this time, they developed splenomegaly and lymphadenopathy, with greater than six-fold increases in cell numbers compared with anti-CD28-treated wild-type mice (data not shown). CTLA-4/B7-1/B7-2 TKO mice treated with anti-CD28 also developed lymphocytic infiltrates and necrosis, in an organ distribution and severity comparable to the CTLA-4 TKO mice (data not shown). No lymphoid or other organ involvement was observed in CTLA-4/B7-1/B7-2 TKO mice receiving control IgG, or wild-type mice receiving anti-CD28 or control IgG. Anti-CD28 administration also markedly increased the fraction of activated T cells in the CTLA-4/B7-1/B7-2 TKO mice compared with wild-type, as determined by CD69 (Fig. 5) and CD62L expression (data not shown). Thus, signaling through CD28 in the CTLA-4/B7-1/B7-2 TKO strain can reproduce the lymphoproliferative disease typical of CTLA-4 TKO mice. The lack of effect of anti-CD28 antibody in wild-type mice in vivo is also noteworthy, demonstrating that engagement of CTLA-4

Figure 3. In the absence of CTLA-4, in vivo T cell activation requires B7-1 or B7-2. Splenocytes were isolated from 2-wk-old mice and double stained with PE-anti-CD3 and FITC-anti-CD69 for analysis by flow cytometry. The percentage shown next to each dot plot indicates the proportion of CD3+ cells that are CD69+. Results are representative of five mice of each strain.

Figure 4. Proliferation of CTLA-4 TKO T cells requires B7-1- or B7-2-mediated costimulation. (A) Proliferative responses of unstimulated splenocytes from 2-wk-old mice of the indicated strain at 24 h are shown. Data are representative of three experiments and the mean of two mice of each strain are shown. (B) Proliferative responses of anti-CD3-stimulated splenocytes from 2-mo-old mice at 48 h are shown. Data are representative of four experiments. The mean of two mice of each strain are shown. Proliferation at all time points was assayed in triplicate, with SD < 15% of the mean.
by endogenous levels of B7 can inhibit the proliferation of T cells receiving a strong positive signal through CD28.

Discussion

The dual specificities of B7-1 and B7-2 for CD28 and CTLA-4 have made it challenging to elucidate physiological interactions in the B7-CD28/CTLA-4 pathway. The uncontrolled T cell activation seen in the CTLA-4−/− mice provides a valuable experimental system for dissecting in vivo functions of B7-1 and B7-2, and for searching for additional members of this family of costimulators. To do this, we have generated mouse strains lacking CTLA-4 and either B7-1, B7-2, or both B7 molecules, and analyzed them for evidence of T cell activation in vivo and in vitro. The phenotypes of the CTLA-4−/−/B7-1−/− and CTLA-4−/−/B7-2−/− strains demonstrate overlapping roles for B7-1 and B7-2 in CD28-mediated signaling, as the presence of either B7-1 or B7-2 is sufficient to produce the CTLA-4−/− phenotype. However, the CTLA-4−/−/B7-1−/− mice have shorter survival and greater T cell activation thanagematched CTLA-4−/−/B7-2−/− mice. These differences may reflect earlier expression and higher cell surface levels of B7-2 compared with B7-1. Since either B7 molecule is capable of producing lymphoproliferation and T cell activation in the CTLA-4−/− mice, it seems unlikely that B7-1 and B7-2 produce fundamentally different signals through CD28.

We thank Abul Abbas for careful review of the manuscript, Vijay K. Kuchroo for the gift of anti-CD28 antibody, Frank Borriello and Elizabeth A. Tivol for initiating mouse breeding, and Sumi Scott for technical assistance.
This work was supported by National Institutes of Health grants K11 AI01212 to D.A. Mandelbrot, AI09709 to A.J. McAdam, and RO1 AI38310, RO1 AI40614, and PO1 AI35297 to A.H. Sharpe.

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Received for publication 23 October 1998.

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