

## CTLA4 mediates antigen-specific apoptosis of human T cells

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**ABSTRACT** The regulation of T cell-mediated immune responses requires a balance between amplification and generation of effector function and subsequent selective termination by clonal deletion. Although apoptosis of previously activated T cells can be induced by signaling of the tumor necrosis factor receptor family, these molecules do not appear to regulate T-cell clonal deletion in an antigen-specific fashion. We demonstrate that cross-linking of the inducible T-cell surface molecule CTLA4 can mediate apoptosis of previously activated human T lymphocytes. This function appears to be antigen-restricted, since a concomitant signal T-cell receptor signal is required. Regulation of this pathway may provide a novel therapeutic strategy to delete antigen-specific activated T cells.

Whereas events regulating the initiation and amplification of T cell-mediated immune responses are well characterized, the cellular interactions and signaling events resulting in antigen-specific peripheral T-cell clonal deletion are ill understood. Following clonal expansion and effector function, previously activated murine T cells are selectively depleted in the periphery (1–4). Several cell surface molecules, including Fas and tumor necrosis factor receptors, induce apoptosis of previously activated T cells (5–8). However, these molecules are not T-lineage-restricted, and there is no evidence that they delete T cells in an antigen-specific manner. The identification of a T-cell antigen-specific apoptotic signal or a T-cell surface-restricted molecule that might associate with apoptotic ligands would be central to our understanding of the regulation of T cell-mediated immunity.

The B7 family of costimulatory molecules delivers a critical costimulatory signal through CD28 on the T cell (9–11). Blockade of CD28 signaling is sufficient to induce anergy (12), and in the absence of interleukin 2 (IL-2), anergized T cells undergo apoptosis (13). In addition to CD28, B7 family members have an alternative T-cell surface receptor, CTLA4 (cytotoxic T lymphocyte-associated protein 4) (14). CTLA4 is T-cell-restricted, appears after T-cell activation (15), and shares 31% overall amino acid identity with CD28 (16). A striking feature of CTLA4 is the total phylogenetic conservation of the cytoplasmic domain (17). Although it has been proposed that CD28 and CTLA4 are functionally redundant, anti-CD28 Fab totally blocks T-cell responses to costimulation by both B7-1 and B7-2 (10, 11). Here we demonstrate that CTLA4 does not provide a redundant CD28-like signal. In contrast, crosslinking of CTLA4 of previously activated T cells results in antigen-specific apoptosis.

### MATERIALS AND METHODS

**Proliferation Assays and Cytokine Detection.** T-cell subsets were obtained from peripheral blood mononuclear cells (PBMC) by selective removal with monoclonal antibodies (mAbs) and immunomagnetic beads. T-cell blasts were isolated after culture in medium containing phytohemagglutinin (PHA) for different

time periods. Alloreactive T-cell clones with specificity for HLA-DR7 were made from HLA-DR7-negative individuals (12). T-cell clones were activated by culture with an *HLA-DR7* homozygous lymphoblastoid line (LBL-DR7). T-cell clones selected did not express either B7-1 or B7-2 either before or after activation. Microtiter plates were coated with anti-CD3 mAb at a concentration of 1  $\mu\text{g}/\text{ml}$ . T cells ( $10^5$  cells per well) were cultured at 37°C for 72 hr in the presence of mAbs (10  $\mu\text{g}/\text{ml}$ ). Where indicated, IL-2 was added (final concentration, 100 units/ml). Thymidine incorporation during the last 16 hr was assessed as an index of mitogenic activity. IL-2 accumulation at 24 hr was assayed by ELISA.

**Transfectants.** Chinese hamster ovary (CHO) cells were transfected with human CD28 cDNA (CHO-CD28). Successful expression of CTLA4 was obtained after the extracellular domain of CTLA4 was PCR-amplified from human CTLA4 cDNA and ligated into pCDM8 vector containing the glycosylphosphatidylinositol anchor of human CD58 (18). CHO-CTLA4 cells were sorted and subcloned by B7-1-Ig fusion protein. COS cells were transfected with cDNA encoding human HLA-DR  $\alpha$  chain (DR $\alpha$ ) and HLA-DR7  $\beta$  chain (together designated transfectant t-DR7) alone or coexpressing DR7 and human B7-1 (t-DR7/B7-1) or human B7-2 (t-DR7/B7-2). NIH 3T3 cells were transfected with cDNA encoding human DR7 and B7-1 (t-DR7/B7-1). Cells were fixed in paraformaldehyde (0.4%) prior to use.

**mAbs.** BALB/c mice were immunized with the recombinant extracellular domain of human CTLA4 or phorbol 12-myristate 13-acetate-activated human PBMC. Three fusions of murine spleen cells and Sp2/0 resulted in 18 cloned mAbs that reacted with recombinant CTLA4 by ELISA. Five of these mAbs were reactive with CHO-CTLA4 cells. Anti-CD28, anti-CD28 Fab, anti-B7-1, anti-B7-2 (B-70; PharMingen), BB1, CTLA4-Ig (13) or control Ig, or anti-CTLA4.1 Fab was added to appropriate cultures (final concentration, 10  $\mu\text{g}/\text{ml}$ ).

**Epitope Mapping.** Epitope mapping was performed by phage display (19).

**Binding Assays.** CTLA4-Ig was bound to plates, and either biotinylated B7-1-Ig or B7-2-Ig fusion protein was evaluated as a binding ligand by ELISA.

### RESULTS

**Anti-CTLA4 mAbs Do Not Costimulate T-Cell Proliferation or IL-2 Secretion.** We generated a panel of anti-CTLA4 mAbs to determine the function of this molecule on resting and previously activated peripheral blood human T cells and T-cell clones. Fig. 1A summarizes the binding of the five anti-CTLA4 mAbs and of a representative anti-CD28 mAb to CHO cells expressing cell surface CD28 or CTLA4. An anti-CD28 mAb

Abbreviations: mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; IL-2, interleukin 2; PHA, phytohemagglutinin; DR7, HLA-DR7; t, transfectant; TCR, T-cell antigen receptor.

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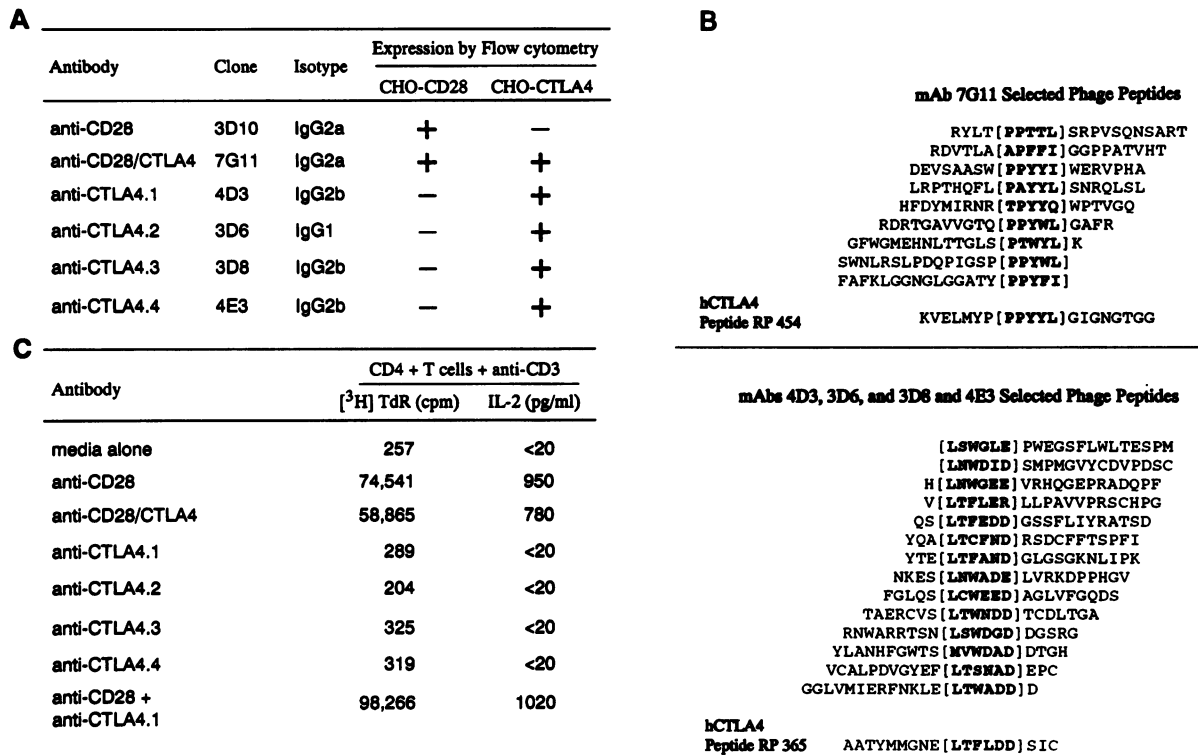


FIG. 1. Surface expression, epitope mapping, and function of anti-CTLA4 mAbs. (A) Isotype and surface expression of a panel of anti-CTLA4 mAbs. (B) Epitope mapping of the anti-CTLA4 mAbs. (C) Costimulation assessed by proliferation and IL-2 accumulation of CD4<sup>+</sup> T cells is provided by anti-CD28 mAb but not by anti-CTLA4 mAb. hCTLA4, human CTLA4; [<sup>3</sup>H]TdR, tritiated thymidine incorporation.

(3D10) bound to CHO-CD28 but not CHO-CTLA4 cells. One mAb (7G11) bound to both CD28- and CTLA4-transfected CHO cells and is henceforth termed anti-CD28/CTLA4 mAb. Four mAbs bound only to CHO-CTLA4 and are termed CTLA4.1 (4D3), CTLA4.2 (3D6), CTLA4.3 (3D8), and CTLA4.4 (4E3). Epitope mapping of the anti-CTLA4 mAbs was performed by phage display and was confirmed with synthetic peptides (19). The epitopes recognized by the anti-CTLA4 mAbs were determined by using a phage display library expressing a random 20-mer amino acid segment and a micropanning protocol (19). Analysis of 20 randomly selected phage from a panning on the anti-CD28/CTLA4 mAb yielded the 9 unique peptide sequences shown in Fig. 1B. All of these sequences contained a core consensus sequence of five residues, Pro-Pro-Tyr-Tyr-(Leu or Ile, where Ile is isoleucine) within which aromatic residues phenylalanine and tryptophane were frequently substituted for tyrosine. The sequence Pro-Pro-Tyr-Tyr-Leu is present within the extracellular domain of CTLA4, within or very close to the CDR3 region, a portion of which is encompassed in synthetic peptide RP 454. The peptide RP 454 was used to confirm the epitope of anti-CD28/CTLA4 mAb and could compete with native CTLA4 for binding of anti-CD28/CTLA4 mAb (data not shown). The sequence identity of CD28 and CTLA4 across this region explains the cross-reactivity observed with this mAb on CHO-CD28 and CHO-CTLA4. The epitopes for mAbs CTLA4.1-CTLA4.4 were determined in separate pannings and were found to be similar for all four mAbs. Forty-two randomly selected phage obtained from panning on all four anti-CTLA4 mAbs yielded 14 unique peptide segments shown in Fig. 1B. From these peptides, the shared epitope was mapped to a stretch of six residues on CTLA4, Leu-Thr-Phe-Leu-Asp-Asp. All four mAbs recognized this core sequence and bound to peptide RP365 derived from the human CTLA4 sequence containing this motif. This shared epitope maps to the CDR2-like region. CTLA4.1 and CTLA4.2 mAbs immunoprecipitate a 41- to 43-kDa protein under nonreducing conditions from the cell surface of activated human peripheral blood T cells and from T-cell clones and immunoprecipitate human

CTLA4 from CTLA4-transfected COS cells (data not shown). Western blotting with the anti-CD28/CTLA4 mAb confirms that the immunoprecipitated protein was CTLA4 and was not recognized by anti-CD28 mAb.

Since the anti-CTLA4 mAbs bound to two distinct antigenic regions on the CTLA4 molecule, we examined whether either of these regions bound B7 family members. CTLA4-Ig was bound to plates, and either biotinylated B7-1-Ig or B7-2-Ig fusion protein was evaluated as a binding ligand. Both B7-1-Ig and B7-2-Ig bound to CTLA4-Ig. However, neither anti-CD28/CTLA4 mAb nor anti-CTLA4.1 mAb could inhibit binding of either B7-1-Ig or B7-2-Ig (data not shown). Similarly, B7-1-Ig did not inhibit binding of anti-CTLA4.1 mAb or anti-CD28/CTLA4 mAb to CTLA4-Ig. Therefore, these antibodies do not bind to the B7-1 or B7-2 binding sites on CTLA4. This result was somewhat surprising since it had been suggested previously that the sequence Met-Tyr-Pro-Pro-Tyr was part of the binding site of both CD28 and CTLA4 to B7-1 (16).

**Anti-CTLA4 mAbs Decrease Proliferation and Induce Apoptosis of Previously Activated T-Cell Clones or T-Cell Blasts.** To determine whether one or more of these mAbs could deliver either a primary or CD28-like costimulatory signal, resting CD4<sup>+</sup> T cells were cultured with antibodies alone or in the presence of submitogenic doses of anti-CD3 mAb. None of these antibodies alone induced proliferation or IL-2 secretion by resting T cells (data not shown). Coculture of resting T cells with anti-CD3 mAb and either anti-CD28 or anti-CD28/CTLA4 mAb resulted in proliferation and IL-2 accumulation (Fig. 1C). In contrast, none of the anti-CTLA4-specific mAbs provided a CD28-like costimulatory signal, even when cross-linked by using a second-step antibody. However, the addition of anti-CTLA4 mAb over a wide variety of different concentrations provided an agonistic signal to that provided by anti-CD3 and anti-CD28 mAbs comparable to published results (15, 20). Similar patterns of results were observed whether the responding population was purified T cells or fractionated CD28<sup>+</sup> or CD8<sup>+</sup> cells (data not shown).

Since CTLA4 is expressed on activated but not resting T cells, we examined whether anti-CTLA4 mAb-mediated costimulation of previously activated T cells might induce proliferation and IL-2 production or, alternatively, might induce apoptosis. HLA-DR7-specific alloreactive T-cell clones were activated by culture for 24 hr with an irradiated *HLA-DR7*<sup>+</sup> homozygous lymphoblastoid cell line (LBL-DR7) that expresses both B7-1 and B7-2. Antigenic stimulation with LBL-DR7 markedly upregulated CTLA4 surface expression on the T-cell clones, but the activated clones do not express B7-1 or B7-2 (data not shown). Activated T-cell clones were rechallenged with NIH 3T3 cells transfected to express HLA-DR7 (t-DR7) either alone or in the presence of anti-CD28 or anti-CTLA4 mAbs. As seen in Fig. 2A, previously activated T-cell clones proliferated modestly in response to challenge with t-DR7 although no IL-2 accumulation was detected. The addition of anti-CD28 or anti-CD28/CTLA4 mAb resulted in significant proliferation and IL-2 accumulation. In contrast, the addition of anti-CTLA4.1 mAb did not stimulate proliferation but instead resulted in significantly decreased proliferation, no detectable IL-2 accumulation (Fig. 2A), and apoptosis (Fig. 3). Greater than 90% of the T-cell clones underwent apoptosis under these culture conditions as assessed by uptake of Hoechst 33342 dye (21). Since all four anti-CTLA4 mAbs shared a common epitope and had identical functional effects, the results in this and in all subsequent experiments are

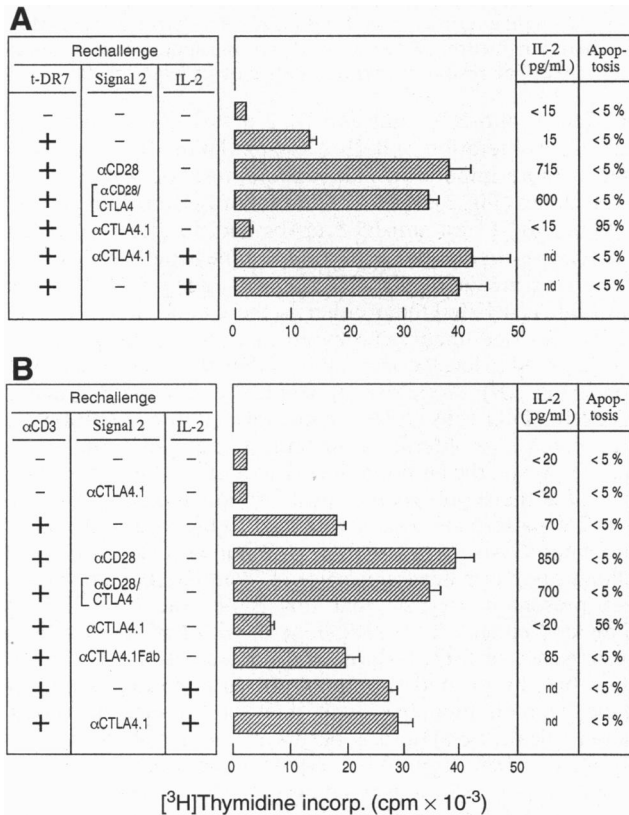


FIG. 2. Anti-CTLA4 mAbs induce decreased proliferation and apoptosis of previously activated T cells. (A) Previously activated alloreactive T-cell clones with specificity for HLA-DR7 were rechallenged with NIH 3T3 cells transfected with cDNA encoding human t-DR7 alone and with mAbs as shown under "Signal 2." The results are representative of six experiments on two T-cell clones. (B) CD4<sup>+</sup> T-cell blasts after culture of PBMC for 4 days in medium containing PHA. Thymidine incorporation (incorp.) was assessed during the last 16 hr of a 48-hr culture. IL-2 accumulation in the supernatant was assessed at 24 hr by ELISA. Apoptosis was assessed by the presence of DNA fragmentation and quantified by flow analysis of uptake of Hoechst 33342 and exclusion of propidium iodide. α, Anti-

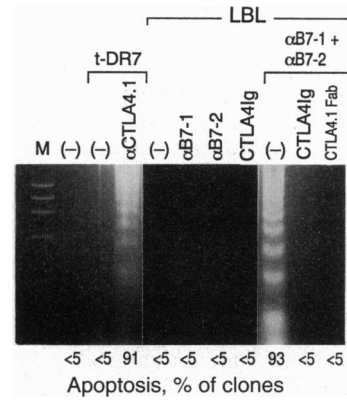


FIG. 3. Alloreactive T-cell clones undergo antigen-specific apoptosis after crosslinking with anti-CTLA4 mAb or a cell surface CTLA4-binding ligand on the surface of a lymphoblastoid cell line. Alloreactive T-cell clones were cultured for 8 hr in the absence (lanes "-") or presence of the indicated stimuli. Cells were washed in PBS and pelleted, DNA was extracted, and the samples were loaded into a 1% agarose gel containing 0.5 μg of ethidium bromide per ml and electrophoresed; DNA was visualized under UV light. Apoptosis was quantified by uptake of Hoechst 33342 and exclusion of propidium iodide. The results are representative of six experiments. Lane M, molecular weight markers (*ϕ*X174 *Hae* III digest). LBL, lymphoblastoid cell line; α, anti-

presented for anti-CTLA4.1 only. Apoptosis was also induced by the anti-CD28/CTLA4 mAb in the presence of anti-CD28 Fab fragments. Functionally this mAb costimulates by crosslinking CD28, resulting in IL-2 production that prevents CTLA4-mediated apoptosis. However, in the presence of anti-CD28 Fab fragments, this mAb crosslinks only CTLA4 and induces apoptosis. Crosslinking an independent epitope on CTLA4 suggests that it is highly unlikely that crosslinking the Leu-Thr-Phe-Leu-Asp-Asp region induces apoptosis purely by cross-reacting with another molecule. The addition of exogenous IL-2 to the wells containing activated T cells with t-DR7 and anti-CTLA4.1 mAb resulted in significant proliferation comparable to that observed in response to t-DR7 and prevented the induction of apoptosis.

Following culture with either t-DR7 or anti-CTLA4.1 mAb for 24 hr, previously activated T-cell clones subsequently proliferated with the addition of exogenous IL-2. However, after culture for 24 hr with the combination of t-DR7 and anti-CTLA4.1 mAb, the T cells did not proliferate with the addition of exogenous IL-2, indicating that these cells had undergone antigen-specific clonal deletion (Table 1). Thus, a signal through the T-cell antigen receptor (TCR) in addition to CTLA4 crosslinking was required to induce apoptosis and clonal deletion.

Table 1. Antigen-specific clonal deletion

Rechallenge conditions*			[ <sup>3</sup> H]Thymidine incorporation,† cpm
t-DR7	Anti-CTLA4.1	IL-2	
-	+	-	26,388 ± 2,153
+	-	-	29,754 ± 1,736
+	+	-	1,370 ± 98
+	+	+	32,593 ± 2,074

Alloreactive T-cell clones were activated by culture with LBL-DR7 cells for 24 hr isolated on Percoll gradients, washed, and then rechallenged for 24 hr under the conditions shown. Cells were washed and cultured in medium containing 100 units of IL-2 per ml for 48 hr, and thymidine incorporation during the last 8 hr of culture was measured. The results are representative of four experiments on two T-cell clones.

\*Prior to washing and 48-hr culture with IL-2.

†In last 8 hr of a 48-hr culture with IL-2.

To determine whether CTLA4 ligation could also induce apoptosis in previously activated normal human T cells, PBMC were cultured with phytohemagglutinin (PHA) for 4 days. CD4<sup>+</sup> T cell blasts were isolated and cocultured with anti-CD3 mAb in the presence of second signals as depicted in Fig. 2B. Anti-CTLA4 mAbs alone had no effect. Anti-CD3 alone induced modest proliferation of PHA-stimulated blasts accompanied by low levels of IL-2 accumulation. The addition of anti-CD28 or anti-CD28/CTLA4 mAbs enhanced both proliferation and IL-2 accumulation. Anti-CD3 in the presence of any of the anti-CTLA4 mAbs also resulted in decreased proliferation, absence of IL-2 accumulation (Fig. 2B), and apoptosis (data not shown). Apoptosis of >50% of the CD4<sup>+</sup> T-cell blasts was detectable as early as 8 hr of culture as assessed by uptake of Hoechst 33342 dye and flow cytometric analysis. Cross-linking of CTLA4 was necessary because anti-CTLA4.1 Fab did not decrease proliferation or induce apoptosis. The addition of exogenous IL-2 to anti-CTLA4.1 mAb protected against apoptosis. Similarly, the addition of anti-CD28 mAb also protected against apoptosis (data not shown). The apoptosis did not appear to be merely as a result of loss of production of IL-2 since CD4<sup>+</sup> PHA-stimulated blasts cultured in medium alone did not undergo apoptosis for periods up to 24 hr, by which time >80% of the cells cultured with anti-CD3 and anti-CTLA4.1 mAbs had undergone apoptosis. The apoptosis induced by CTLA4 crosslinking appeared to be specific, since anti-CD45, anti-CD45RA, anti-CD45RO, anti-CD4, anti-CD5, or anti-CD6 mAb crosslinking under identical culture conditions did not induce decreased proliferation or apoptosis in any of six experiments performed (data not shown). Similar levels of apoptosis were also observed in isolated CD28<sup>+</sup> (apoptosis observed in >70% by 8 hr) and CD8<sup>+</sup> (apoptosis in >60% by 8 hr) or unfractionated PHA-stimulated blasts (apoptosis observed in >60% by 8 hr) examined under the identical culture conditions (data not shown).

**B7-1 and B7-2 Do Not Induce CTLA4-Mediated Apoptosis.** Since CTLA4-mediated costimulation induced apoptosis of previously activated normal T cells and alloreactive T-cell clones, we sought to determine whether the known CTLA4-binding ligands mediated this function. Activated T-cell clones were rechallenged with COS cell transfectants t-DR7, t-DR7/B7-1, or t-DR7/B7-2. t-DR7 plus anti-CTLA4.1 suppressed proliferation of the T-cell clone and induced apoptosis (Fig. 4A). Both t-DR7/B7-1 and t-DR7/B7-2 augmented proliferation, and apoptosis was not observed. Addition of anti-CD28 Fab blocked the augmented proliferation induced by B7-1 and B7-2, demonstrating that the proliferative signal was indeed mediated via CD28. However, under these circumstances where binding to CD28 is blocked, binding of B7-1 or B7-2 to CTLA4 neither decreased proliferation nor induced apoptosis. Identical results were observed with PHA-activated human T cells (data not shown).

**An Alternative CTLA4 Ligand Induces Antigen-Specific Apoptosis of Previously Activated T Cells.** Since the two molecularly cloned CTLA4 ligands did not mediate antigen-specific clonal deletion, we attempted to determine whether alternative CTLA4 ligands might mediate this effect. Activated T-cell clones were rechallenged with LBL-DR7 cells alone or in the presence of mAb and fusion proteins as shown in Fig. 4B. mAbs were then added to block B7-mediated costimulation and IL-2 accumulation, since these ligands provided a positive signal that blocked anti-CTLA4 mAb-induced apoptosis. Blocking mAbs directed against B7-1, B7-2, and B7-3 (BB1) individually suppressed LBL-DR7-induced proliferation by up to 25%. Addition of CTLA4-Ig suppressed proliferation by 50% and totally blocked IL-2 accumulation, with the resulting proliferation comparable to that seen with t-DR7 alone. The addition of control Ig had no effect. In contrast, simultaneous

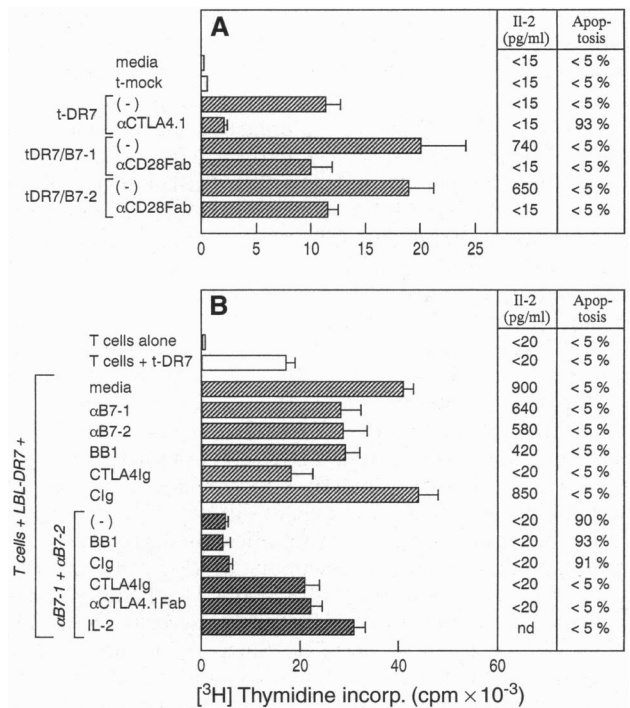


FIG. 4. CTLA4 crosslinking induces decreased proliferation and apoptosis of previously activated alloreactive T-cell clones. Thymidine incorporation (incorp.), IL-2 accumulation, and apoptosis are assessed as in Fig. 2. The results are representative of six experiments.

addition of anti-B7-1 and anti-B7-2 mAbs significantly suppressed proliferation and IL-2 accumulation (Fig. 4B) and, what is more important, induced apoptosis of >90% of the T-cell clones (Fig. 3). Further addition of BB1 mAb or control Ig to anti-B7-1 and anti-B7-2 mAbs had no effect on either proliferation or apoptosis. To determine whether this apoptosis was mediated via CTLA4, we added CTLA4-Ig or anti-CTLA4.1 Fab. Under either of these conditions, <5% of the T-cell clone underwent apoptosis (Fig. 3), and proliferation returned to levels comparable to that observed with t-DR7 alone (Fig. 4B). Apoptosis appeared to be antigen-specific, since LBL-DR1 cells under the identical culture condition did not decrease proliferation or induce apoptosis (data not shown). Again, the addition of IL-2 to anti-B7-1 and anti-B7-2 abrogated the suppression of proliferation and apoptosis. An identical pattern of results was again observed with PHA-stimulated blasts (data not shown). Whereas it is possible that cellular apoptosis occurs because of "lymphokine blockade," data presented suggests that this is not the case. T cells stimulated either with t-DR7 alone or with LBL-DR7 cells in the presence of CTLA4-Ig have no detectable IL-2 accumulation but do not undergo apoptosis. In addition, secondary challenge with medium alone does not induce apoptosis. Cellular death by apoptosis occurs in the T-cell clones only after 48 hr when cultured in medium alone, whereas CTLA4-mediated apoptosis is observed by 8 hr. CTLA4-Ig blocks both B7-1 and B7-2 yet does not have the same effect as the anti-B7-1 and anti-B7-2 mAbs. This suggests that it is not simply the absence of IL-2 production that results in apoptosis but rather binding to an alternative CTLA4 ligand that provides a positive apoptotic signal.

Taken together, these results demonstrate that a physiologic ligand for CTLA4 that mediates antigen-specific T-cell apoptosis: (i) is expressed on LBL-DR7 cells, (ii) is neither B7-1 nor B7-2, (iii) is a CTLA4-binding ligand because CTLA4-Ig blocked apoptosis, and (iv) signals through CTLA4 since anti-CTLA4.1 Fab also blocks apoptosis. These results are consistent with the hypothesis that CTLA4 provides not a redundant

CD28-like costimulatory pathway but rather a distinct signaling pathway capable, under appropriate conditions, of clonally deleting previously activated T cells.

### DISCUSSION

CTLA4 is a T cell-restricted cell surface molecule induced with TCR or CD28 activation. Its cytoplasmic tail is 100% conserved among chicken, mouse, and man, suggesting that this molecule is likely to mediate an important functional event. The present results show that, whereas CD28 costimulation by B7-1, B7-2, or anti-CD28 mAb induces secretion of multiple cytokines and upregulates IL-2 receptor expression on resting or previously activated T cells, under the identical conditions CTLA4 ligation provides no such stimulus. In contrast, concomitant antigen receptor signaling and CTLA4 crosslinking result in cellular apoptosis. Therefore, CTLA4 is not associated with a redundant CD28-like costimulatory pathway but rather a distinct signaling pathway capable of clonally deleting previously activated T cells when TCR signaling is not accompanied by significant IL-2 accumulation.

During an ongoing immune response, there is a balance between signals mediating activation and amplification and those that subsequently induce antigen-specific cellular deletion. Either cross-linking of CD28 or the common binding region of CD28/CTLA4 by mAbs or by their natural ligands B7-1 or B7-2 provides a positive costimulatory signal resulting in IL-2 accumulation. Our results suggest that signals that induce IL-2 accumulation are dominant, since they amplify the immune response and protect an ongoing immune response from CTLA4-mediated apoptosis. Since B7-1 and B7-2 are ligands for both CD28 and CTLA4, their function should be to mediate amplification rather than cellular deletion. During this interval of amplification, crosslinking of CTLA4 does not mediate apoptosis but, in contrast, can provide a weak synergistic costimulatory signal to CD28. After T-cell activation, CD28 engagement by B7-1 down-regulates CD28 synthesis and function as CTLA4 expression increases (22). Under conditions where the proliferative response is waning, crosslinking of CTLA4 in the absence of CD28-mediated costimulation can then induce cellular deletion of previously activated cells. The functional capacity to either costimulate or induce apoptosis depending on the state of activation of a cell is highly reminiscent of the functional repertoire of members of the tumor necrosis factor (TNF) receptor family (23). However, unlike the apoptosis induced by members of the TNF receptor family, CTLA4 crosslinking appears to mediate antigen-specific clonal deletion, since it requires a concomitant signal through the TCR. The mechanism whereby CD28-mediated signaling prevents apoptosis is unknown, but since this apoptosis can also be prevented by the addition of exogenous IL-2, it suggests that a viability signal is mediated through IL-2 receptor signaling.

Following activation of T-cell clones, at a time when CD28 expression is decreased and CTLA4 expression is maximal, neither t-DR7/B7-1 nor t-DR7/B7-2 induced apoptosis but rather provided a positive signal. Blocking B7-1 or B7-2 binding to CD28 with anti-CD28 Fab did not mimic the effect of crosslinking CTLA4 with CTLA4.1 mAb. These results suggest that neither B7-1 nor B7-2 induce CTLA4-mediated apoptosis. LBL-DR7 cells induce a secondary proliferative response of previously activated T-cell clones. The addition of anti-B7-1, anti-B7-2, or BB1 mAbs reduced proliferation and IL-2 production, but no apoptosis was observed. Similarly, CTLA4-Ig, which blocks all members of the B7 family, reduced proliferation and detectable IL-2 accumulation, but again no apoptosis was observed. In contrast, the identical LBL-DR7 cells in the presence of anti-B7-1 and anti-B7-2 mAbs inhibited proliferation and IL-2 production and, more importantly, induced >90% apoptosis of the T-cell clone. Subsequent blocking experiments demonstrate that the apoptotic ligand binds to CTLA4-Ig. Moreover, apoptosis is completely abro-

gated by a Fab fragment of the CTLA4.1 mAb, demonstrating that the natural ligand binds at or close to the same binding site as the CTLA4.1 mAb. Therefore, these results demonstrate that the natural ligand that can induce apoptosis is a CTLA4-binding molecule and binds to the identical site that the CTLA4.1 mAb bound on CTLA4.

There is no evidence that this ligand is a member of the B7 costimulatory family, but a potential candidate might be B7-3, a CTLA4-binding ligand that costimulates without inducing IL-2 accumulation (24). However, anti-B7-3 (BB1) mAb did not block CTLA4-mediated apoptosis. Regardless of the mechanism, the recognition of an antigen-specific pathway for clonal deletion of previously activated T cells would obviously have great potential for the termination of immune responses. Clinical manipulation of the balance between T-cell activation and subsequent clonal deletion, with particular relevance to autoimmunity and organ transplantation, should require both inhibition of positive signals leading to T-cell amplification and antigen-specific signaling with CTLA4 crosslinking.

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