# CD28 and CTLA-4 Have Opposing Effects on the Response of T cells to Stimulation

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## Summary

The importance of the B7/CD28/CTLA-4 molecules has been established in studies of antigen-presenting cell–derived B7 and its interaction with the T cell costimulatory molecule CD28. CTLA-4, a T cell surface glycoprotein that is related to CD28, can also interact with B7-1 and B7-2. However, less is known about the function of CTLA-4, which is expressed at highest levels after activation. We have generated an antibody to CTLA-4 to investigate the consequences of engagement of this molecule in a carefully defined system using highly purified T cells. We show here that the presence of low levels of B7-2 on freshly explanted T cells can partially inhibit T cell proliferation, and this inhibition is mediated by interactions with CTLA-4. Cross-linking of CTLA-4 together with the TCR and CD28 strongly inhibits proliferation and IL-2 secretion by T cells. Finally, results show that CD28 and CTLA-4 deliver opposing signals that appear to be integrated by the T cell in determining the response to activation. These data strongly suggest that the outcome of T cell antigen receptor stimulation is regulated by CD28 costimulatory signals, as well as inhibitory signals derived from CTLA-4.

Recent work has demonstrated that CD28, a protein expressed on resting and activated cells, is the major costimulatory molecule for proliferation of T cells (1–3). CD28 engagement via antibodies augments the proliferation of T cells in response to immobilized anti-TCR antibodies (4). Additionally, antibody engagement can supply costimulation to T cells encountering APCs deficient in costimulation (4, 5) and prevents the resultant anergic state that otherwise occurs in long-term clones (4). Finally, Fab fragments of anti-CD28 can fully block proliferation by costimulation competent APCs (4).

Several lines of evidence indicate that B7-1 (CD80) and B7-2 (CD86) (6) are the major costimulatory ligands on the APC. First, a chimeric fusion protein of CD28 binds B7-1 and B7-2 (7, 8). Second, anti-B7 antibodies block T cell activation by a variety of APCs (9, 10). Finally, induction of expression of B7-1 or B7-2 by transfection with cDNAs confers costimulatory activity on cells that do not otherwise provide costimulation (11–14). Interestingly, APCs and especially dendritic cells, which are thought to be involved in the early phases of T cells activation, express moderate levels of functional B7-2 without activation (6, 15). These levels increase nearly 100-fold with overnight activation, enhancing their APC function.

B7-1 and B7-2 also bind CTLA-4, a close relative of CD28. Chimeric fusion proteins consisting of the ectodomain of CTLA-4 bind B7-1 and B7-2 (8, 16–18) and can block T cell activation by costimulation competent accessory cells (10, 12). Notably, studies with soluble fusion protein indicate that CTLA-4 binds both B7 family members with an affinity ~20-fold higher than that of CD28. This higher affinity probably accounts for the ability of the CTLA-4 Ig fusion protein to block costimulation in vitro (10, 12) and to suppress graft rejection and antibody production in vivo (19, 20).

While the ability of CTLA-4 fusion proteins to bind CD28 ligands and block T cell activation is clear, the function of the native molecule has been obscure. Originally identified as cDNA cloned from a subtracted CTL clone library (21), CTLA-4 is homologous to CD28, especially in the extracellular domain, and both contain a conserved sequence motif, MYPPY, thought to be involved in B7 binding (22). Recent work has shown that CTLA-4 mRNA is expressed within a few hours of activation (23). Studies with mAbs to both human and mouse CTLA-4 demonstrated surface expression within 48 h of activation. However, functional studies have led to different conclusions about its role in activation. Linsley et al. in a study of human T cells found that anti–CTLA-4 antibodies enhanced proliferation of T cells activated with anti-CD3 and anti-CD28, suggesting that the function of CTLA-4 was to augment or sustain costimulation (24, 25). Walunas et al. found that both intact and monovalent fragments of antibodies to mouse CTLA-4 enhanced T cell responses in allogeneic MLR, but that intact antibody inhibited proliferation under conditions where Fc receptor cross-linking was provided (26). These results suggest that CTLA-4 might play a role in negative regulation of T cell activation.

We describe here an analysis of CD28 and CTLA-4 signaling on highly purified T cells, noting the presence of B7 on the T cells themselves. The results indicate that the two molecules have opposing effects on lymphokine production and proliferation, and that the outcome of T cell activation is determined by integration of signals transduced by these two molecules.

## Materials and Methods

*Immunization and Hybridoma Production.* 6-wk-old golden Syrian hamsters received five footpad injections of 50 μl (packed volume) heat-killed *Staphylococcus* A bacteria coated with ~100 μg CTLA-4Ig (27) and suspended in 0.2 ml PBS. 3 d after the final injection, draining lymph nodes were removed, and lymphocytes were isolated and fused with the P3X3.Ag8.653 myeloma line using a standard polyethylene glycol fusion technique (28). Hybridoma supernatants were tested for reactivity to CTLA-4 Ig and for a lack of reactivity to CD4 Ig by ELISA (29). Hybridomas from positive wells were repetitively cloned by limiting dilution in the presence of irradiated mouse thymocyte feeder layers. Antibody 9H10 was specific for CTLA-4 by three criteria: (*a*) Reactivity against CTLA-4 Ig but not CD4 Ig; (*b*) the ability to block CTLA-4 Ig binding to B7 transfectants; (*c*) the ability to stain activated T cells but not freshly isolated T cells; and (*d*) the ability to stain a CTLA-4 transfectant but not control transfectants.

*Antibodies.* Antibodies used include anti-CD3 clone 500A2 (30), anti-CD28 clone 37.51 (31), anti-B7-1 clone 1610A (9), anti-B7-2 (17), anti-Vγ3 clone 536 (32), anti-class II MHC clone 28-16-8s (33), and anti-$IA^{d/b}$ clone BP107 (34). Conjugates of these antibodies were prepared in our laboratory. PE, biotin, and FITC conjugates of anti-CD4 and anti-CD8 were purchased from CALTAG Laboratories (South San Francisco, CA) and PharMingen (San Diego, CA).

*T Cell Activation Cultures.* Spleens from 4–6-wk-old BALB/c mice were harvested and minced, and suspensions were treated with Geys RBC lysis solution (35). Cells were cultured in RPMI containing 10% FCS and soluble anti-CD3 antibody at 10 μg/ml.

*Flow Cytometry.* $2 \times 10^5$ cells were suspended in 10 μl ice-cold PBS/1% calf serum/0.05% sodium azide. Antibodies were added for 30 min followed by two 4-ml washes in PBS/calf serum/sodium azide. Data were acquired on a FACScan® (Becton Dickinson and Co. (Mountain View, CA) and the LYSIS II program was used to electronically gate on relevant populations.

*Proliferation Assays.* LN cells were isolated from 6–8-wk-old BALB/c mice (Charles River Laboratories, Wilmington, MA). Isolated lymphocytes were obtained by mincing and filtration through nylon sieves. Cells were then treated with anti–class II antibodies 28.16.8s and BP107 and a mixture of rabbit and guinea pig complement (Accurate Chemical and Scientific Corp., Westbury, NY). Viable cells were isolated over lympholyte 1.119 (Sigma Chemical Co., St. Louis, MO) and residual Ig-positive cells were removed by repetitive panning on rabbit anti–mouse IgG coated tissue culture plates. Typical preparations analyzed by FACS® were typically found to be 99% $Thy1.2^+$ with <0.5% B220-positive cells. Round-bottomed 96-well plates were used for all assays. Where indicated, wells were coated with anti-CD3 at 0.1 μg/ml in 50-μl vol for 2 h at 37°C, then washed extensively and blocked for 30 min at 37°C with complete RPMI 1640. T cells were added at $10^5$ per well in 200 μl complete RPMI 1640. For soluble assays, anti-CD28 was added at a 1:1,000 dilution of ascites, anti–CTLA-4 was added at 10 μg/ml, and B7 antibodies were added at 2.5 μg/ml and 10 μg/ml for anti–B7-1 and anti–B7-2, respectively. For cross-linking assays, anti-CD8 was added at 4 μg/ml, anti-CD3 was added at 5 μg/ml, anti–CTLA-4 was added at 20 μg/ml, and anti-Vγ3 control antibody F536 was added to produce a constant total antibody concentration of 30 μg/ml. Polyclonal goat anti–hamster antibody (Pierce Chemical Co., Rockford, IL) was added at a final concentration of 20 μg/ml. All cultures were incubated at 37°C for 72 h, then pulsed with 1 μCi of [$^3$H]thymidine for an additional 16 h before harvesting.

*Antibody Incorporation onto Latex Microspheres.* Sulfate polystyrene latex microspheres of 5 ± 0.1 μM mean diameter were obtained from Interfacial Dynamics Corporation (Portland, OR). Approximately $1 \times 10^7$ beads per ml were suspended in PBS and the indicated antibodies were incubated for 1.5 h at 37°C. Anti-CD3 was added at 1 μg/ml to deliver the antigen-specific signal, and binding solutions were normalized with control antibody 536 to maintain a constant total antibody concentration of 5 μg/ml during binding. Previous data have shown maximal binding is achieved when this concentration is added per $10^7$ particles (36). Beads were washed extensively with PBS and resuspended in 1 ml RPMI–10% FCS and allowed to block for at least 30 min at room temperature. $10^5$ cells were incubated in 96-well cultures with $10^5$ each of the indicated bead preparation.

## Results

*Activation Induces Expression of CTLA-4 on Both $CD4^+$ and $CD8^+$ T cells.* Antibody 9H10 was used to assess CTLA-4 expression on freshly isolated and activated T cells. As shown in Fig. 1 *A*, CTLA-4 was undetectable on freshly isolated T cells. CTLA-4 was readily detected on T cells 48 h after stimulation by addition of anti-CD3 to splenocytes, and it was returned to resting levels by 72 h. CD28 expression was not greatly altered by stimulation. CTLA-4 was expressed by both $CD4^+$ and $CD8^+$ T cells, with significantly higher levels on the latter.

*Blockade of CTLA-4 or B7-1/2 Enhances Anti-CD28 Costimulation.* To determine the functional consequences of CTLA-4 engagement, soluble anti–CTLA-4 or anti-CD28 antibodies were added singly or together to cultures of purified T cells exposed to immobilized anti-CD3. As shown in Fig. 2, while anti-CD28 greatly enhances the minimal proliferation induced by CD3 stimulation, anti–CTLA-4 has no effect. This suggests that CTLA-4 does not function as an alternate costimulatory receptor. Despite its lack of costimulatory activity on its own, anti–CTLA-4 markedly increases T cell proliferation when given together with anti-CD28.

At least two mechanisms could account for the potentiating effects of anti–CTLA-4: enhancement of activation signals or removal of inhibitory signals. Given the observation that anti–CTLA-4 lacked costimulatory activity on its own, we favored the latter possibility—that blockade of CTLA-4 by the antibody might prevent interaction of CTLA-4 on the T cell surface with B7-1 and/or B7-2 and thus prevent delivery of an inhibitory signal. This is further suggested by the observation that anti–CTLA-4 Fab fragments have similar effects as anti–CTLA-4 mAbs in this assay system (data not shown). Since the cells used in our experiments were >99% $Thy1^+$ and contained no detectable MHC class-$II^+$ or $B220^+$ cells,

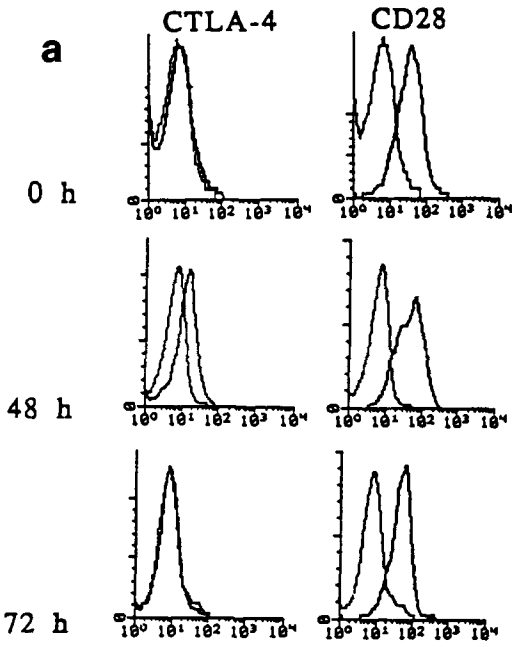


**Figure 1.** Expression of CTLA-4 and CD28 on resting and activated splenic T cells. BALB/c splenic cell suspensions ($2 \times 10^5$/ml) were stimulated in vitro with 10 $\mu$g/ml soluble anti-CD3. (*a*) Cells were double stained with Thy1.2PE and either hamster Ig-control FITC, anti-CTLA-4 FITC, or anti-CD28 FITC. Data were electronically gated for Thy1.2-positive cells; CTLA-4 and CD28 expressions are shown on freshly explanted cells and after 48- and 72-h incubations with anti-CD3. (*b*) 48-h cultures were stained for anti-CD4 biotin or anti-CD8 biotin followed by avidin tricolor and FITCylated irrelevant or CTLA-4 antibodies. Subpopulation-gated data show modestly higher CTLA-4 expression on CD8 populations.

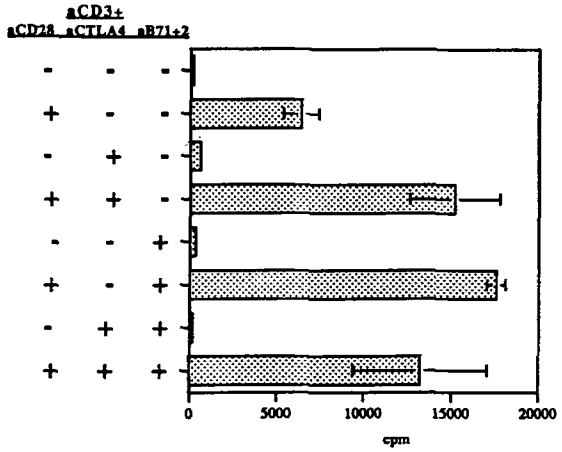


**Figure 2.** Influence of anti-CTLA-4 and anti-B7 antibodies on proliferative responses of purified lymph node T cells. $10^5$ BALB/c LN T cells were cultured for 72 h in 96-well microwells coated with 0.1 $\mu$g/ml anti-CD3. Anti-CD28 was used at a 1:1,000 dilution of ascites and B7 antibodies were used at 2.5 and 10 $\mu$g/ml for anti-B7-1 and anti-B7-2, respectively. Anti-CTLA-4 antibody 9H10 was added at 10 $\mu$g/ml.

this possibility would require that the T cells themselves provide a source of ligand. As shown in Fig. 3, flow cytometric analysis revealed that the freshly isolated T cells did indeed express significant levels of B7-2 and trace levels of B7-1.

To determine the functional consequence of B7 expression by T cells in our assay system, we determined the effects of anti-B7 antibodies on CD28-mediated costimulation. As shown in Fig. 2, anti-B7 antibodies by themselves had no significant effect on anti-CD3-induced T cell proliferation. The addition of anti-B7 antibodies to cultures containing anti-CD28 resulted in a threefold increase in proliferation over that obtained with anti-CD28 alone. A similar increase in proliferation was obtained when chimeric CTLA-4 Ig instead of anti-B7 antibodies was added to block B7 interactions (data not shown). The magnitude of the increase was similar to that obtained when anti-CTLA-4 is added to CD28-treated cells in the absence of B7 blockade. The addition of anti-CTLA-4 to cultures in which anti-B7 antibodies are present results in no further increase in CD28 costimulation; indeed, a slight but reproducible decrease is observed.

Together, these results suggest that T cells express B7 at levels that are insufficient to provide costimulation via CD28 engagement in the assay system used. However, perhaps because of the fact that CTLA-4 has a much higher affinity than CD28 for B7 binding, these levels are sufficient to generate a signal that at least partially inhibits activation. Blockade of the CTLA-4/B7 interaction with either anti-CTLA-4 or anti-B7 antibodies removes the inhibitory signal, resulting in an increase of the costimulatory effect of CD28 ligation.

*Cross-linking of CTLA-4 with the TCR and CD28 Inhibits T Cell Proliferation and IL-2 Production.* The results shown

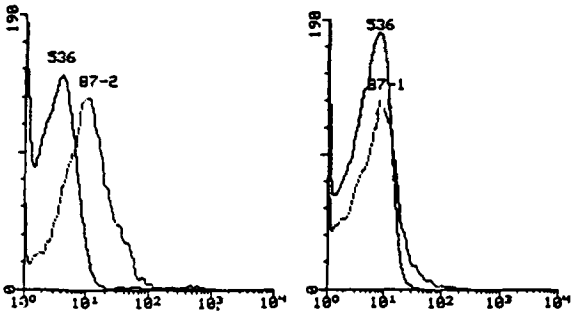


**Figure 3.** Expression of B7-2 and B7-1 on purified, freshly isolated lymph node T cells. Cells that were >99% Thy1.2$^+$ were isolated and stained with GL1 (anti-B7-2), 1610A (anti-B7-1), or irrelevant antibodies.

in Fig. 2 suggested that soluble, bivalent anti-CTLA-4 antibody was effective in blocking B7-mediated signals, but was inefficient in providing signals. We next examined the effects of using anti-hamster Ig to cross-link CD3, CD28, and CTLA-4 singly or together. As shown in Fig. 4, no proliferation was obtained when CD3, CD28, or CTLA-4 were cross-linked individually. As expected, cross-linking of CD3 together with CD28 resulted in potent costimulation, while cross-linking of CD3 and CTLA-4 had no effect. Co-cross-linking of CTLA-4 together with CD3 and CD28 consistently resulted in a 5- to 10-fold reduction in proliferation. This inhibition was largely reversed by the addition of IL-2 to the cultures, suggesting that the effect is not caused by toxicity. Finally, cross-linking of CTLA-4 with CD3 and CD28 also resulted in a profound decrease in IL-2 production in the cultures (Fig. 4 *B*). These results demonstrate that CTLA-4 can deliver signals that inhibit T cell responses to TCR ligation, and that the effects observed in the experiment presented in Fig. 2 most likely result from perturbation of B7/CTLA-4 interactions.

*CD28 and CTLA-4 Deliver Quantitatively Opposing Signals.* The preceding data indicate that CTLA-4 cross-linking in the presence of CD28 signaling can inhibit IL-2 secretion and proliferation. We next sought to determine whether signaling above the threshold for CTLA-4 inhibition is independent of the magnitude of CD28 costimulation, or whether the threshold increases as CD28 signaling increases. To address this issue, T cells were stimulated by incubation with polystyrene beads coated with a constant amount of anti-CD3 and varying amounts of anti-CD28 and anti-CTLA-4. As shown in Fig. 5, costimulation with increasing amounts of anti-CD28 in the absence of anti-CTLA-4 resulted in a gradual increase in proliferation, reaching at the highest dose a 1,500-fold increase over that obtained with anti-CD3 alone. The addition of increasing amounts of anti-CTLA-4 reduced that proliferation in a stepwise manner at all doses of anti-CD28. These results suggest that T cells integrate signals from CD28 with signals from CTLA-4, and the balance of these signals regulates the magnitude of the response to TCR ligation.

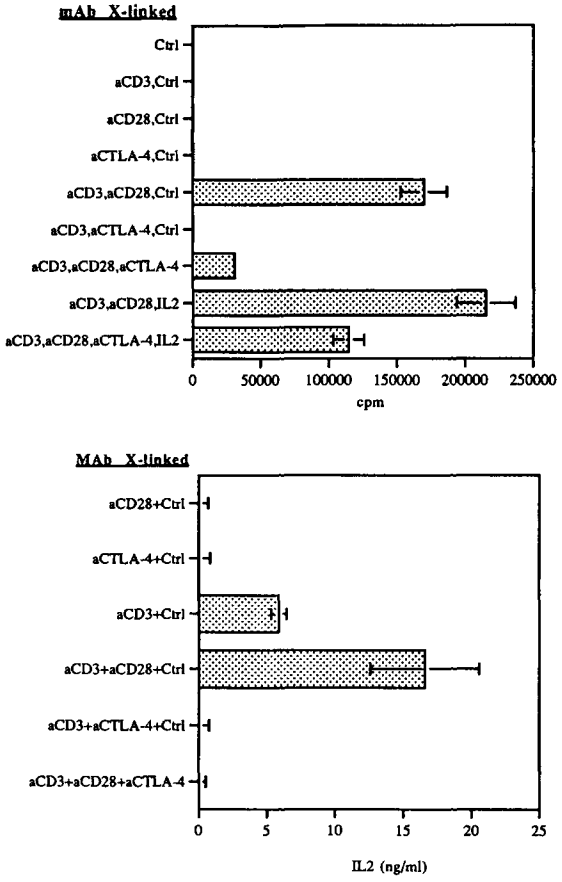


**Figure 4.** Cross-linked anti-CTLA-4 can diminish both proliferation and lymphokine production by purified LN T cells. $10^5$ BALB/c LN T cells were cultured with the indicated hamster antibodies together with control hamster antibodies. Anti-hamster Ig antibody was added at 20 $\mu$g/ml to cross-link. Where indicated, anti-CD3 was added at 5 $\mu$g/ml, anti-CD28 was added at 4 $\mu$g/ml, anti-CTLA-4 was added at 20 $\mu$g/ml, and control was added to normalize antibody concentration at 30 $\mu$g/ml. (*a*) Cells were cultured for 72 h, pulsed with 1 $\mu$Ci [$^3$H]thymidine, and harvested after an additional 16 h. (*b*) Supernatants were removed and analyzed for IL-2 production at 48 h using an ELISA detection system.

## Discussion

The results presented here clearly demonstrate that CTLA-4 does not serve as a functional alternative to CD28 in providing costimulatory signals to T cells. This finding is in agreement with earlier studies showing that CTLA-4 did not replace CD28 function in CD28 mutant mice (37). The finding that anti-CTLA-4 increases proliferation of T cells activated by anti-CD3 and anti-CD28 is in agreement with the results of Linsley et al. (24). However, the fact that a similar result is obtained when blocking antibodies to B7 are included suggests that this apparent cooperativity of CTLA-4 is in fact a result of removal of preexisting inhibitory B7-CTLA-4 in-

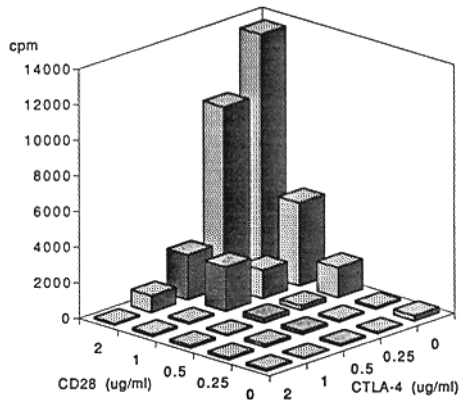


**Figure 5.** Proliferation in response to a constant CD3 signal is affected by the relative concentrations of CD28 and CTLA-4 signals. $10^7$ 5 $\mu$M microspheres were coated with 1 $\mu$g/ml of anti-CD3, the indicated concentrations of anti-CD28 and anti-CTLA-4, and control hamster Ig constituting a total antibody concentration of 5 $\mu$g/ml. $10^5$ coated spheres were incubated in 96-well cultures with $10^5$ purified LN T cells for 72 h, pulsed with 1 $\mu$Ci [$^3$H]thymidine, and harvested after an additional 16 h.

teractions by the soluble CTLA-4 antibodies rather than a synergism between the two antibody-generated signals. The ability of CTLA-4 to directly signal is supported by the fact that cross-linking of anti–CTLA-4, either with second antibody or by presentation immobilized on beads, results in inhibition of both IL-2 production and proliferation. This direct demonstration of signaling by CTLA-4 supports the report of Walunas et al. (26) that CTLA-4 is a negative regulator of T cell activation.

Our demonstration of a dynamic competition between CD28 and CTLA-4 indicates that in addition to CD3 and CD28 signal integration, there exists an integration point for CTLA-4–derived signals. At present there is little knowledge of the biochemical events that follow CD28 or CTLA-4 ligation. There have been reports that CD28 stimulation results in induction of protein tyrosine kinase activity (38), and recent evidence suggest the Tec family kinase ITK represents one associated protein kinase (39). In addition, it has been demonstrated that phosphoinositides accumulate in T cells stimulated by ligation of CD28 with B7-1, suggesting an involvement of phosphatidylinositol 3′-kinase (PI3K)[1] with CD28 (40). In this regard, it is of interest that the cytoplasmic domains of both CD28 and CTLA-4 contain the sequence YM/VXM, a motif found in several growth factor receptors that associate with PI3K (41, 42). Several recent reports have documented a stimulation-induced association between CD28 and PI3K, and it has been reported that mutation of the PI3K-binding motif destroys the costimulatory activity of CD28 (43–47). These findings strongly suggest that binding of PI3K plays an important role in CD28 signaling. With respect to CTLA-4, however, there have been contradictory findings. Whereas a chimeric protein containing the cytoplasmic domain of CTLA-4 was unable to bind PI3K (46), another study reported the coprecipitation of PI3K activity with CTLA-4 (48). In any event, these findings raise the possibility that CD28 and CTLA-4 might compete for PI3K and affect its role in subsequent signal transduction.

It is also possible that CD28 and CTLA-4 signals might intersect at later stages in the pathway. It has been demonstrated that the CD28 and CD3 pathways intersect at the level of the MAP kinase JNK (49). CTLA-4 might in someway interfere with this coupling, thus preventing costimulation. Finally, it is possible that CTLA-4 signals might interfere with those of CD28 even further downstream by interfering with IL-2 transcriptions or mRNA stabilization (46, 50).

Our results further suggest that regulation of the outcome of T cell stimulation is a complex process with regard to events at the cell surface. It is clear that in the absence of costimulatory signals provided by the B7 family, T cells do not proliferate. It appears that even small amounts of B7, such as those present on T cells themselves, are ineffectual in supporting CD28-mediated costimulation of anti-CD3 responses. This appears to be less a consequence of the absence of CD28 signal being delivered, but rather a result of an inhibitory signal delivered through CTLA-4. This implies that either CTLA-4 is quickly expressed after activation and aborts the response, or that CTLA-4 is expressed at functionally significant levels on resting T cells. At higher levels of B7 expression, as might be encountered on activated dendritic cells and activated B cells, CTLA-4 expression on the T cells might become limiting, and the costimulation provided by CD28 becomes dominant. As expression of CTLA-4 rises after activation, the signals generated through CTLA-4 might become dominant and terminate the response. Decay of CTLA-4 expression with time would allow the T cell to return to a state where the CD28 costimulatory signal would predominate. In any event, accumulating evidence suggests that in addition to antigen receptor and CD28-mediated signals, a third signal, provided by CTLA-4, is important in determining the outcome of T cell activation.

[1] *Abbreviation used in this paper:* PI3K, phosphatidylinositol 3′-kinase.



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