**Summary**

Functional interactions between T and B lymphocytes are necessary for optimal activation of an immune response. Recently, the T lymphocyte receptor CD28 was shown to bind the B7 counter-receptor on activated B lymphocytes, and subsequently to costimulate interleukin 2 production and T cell proliferation. CTLA-4 is a predicted membrane receptor from cytotoxic T cells that is homologous to CD28 and whose gene maps to the same chromosomal band as the gene for CD28. It is not known, however, if CD28 and CTLA-4 also share functional properties. To investigate functional properties of CTLA-4, we have produced a soluble genetic fusion between the extracellular domain of CTLA-4 and an immunoglobulin Cy chain. Here, we show that the fusion protein encoded by this construct, CTLA4Ig, bound specifically to B7-transfected Chinese hamster ovary cells and to lymphoblastoid cells. CTLA4Ig also immunoprecipitated B7 from cell surface 125I-labeled extracts of these cells. The avidity of 125I-labeled B7Ig fusion protein for immobilized CTLA4Ig was estimated (Kd ~12 nM). Finally, we show that CTLA4Ig was a potent inhibitor of in vitro immune responses dependent upon cellular interactions between T and B lymphocytes. These findings provide direct evidence that, like its structural homologue CD28, CTLA-4 is able to bind the B7 counter-receptor on activated B cells. Lymphocyte interactions involving the B7 counter-receptor are functionally important for alloantigen responses in vitro.

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The hallmark of a vertebrate immune system is the ability to discriminate between self and nonself. This property has lead to the evolution of a system requiring multiple signals to achieve optimal activation of immunocompetent cells (1). It was proposed >20 yr ago that B lymphocyte activation requires two signals (2). Now it is believed that all lymphocytes require both an antigen-specific signal, as well as a second nonspecific one (1).

The signals required for a Th cell antigenic response are usually provided by APC. The first signal is initiated by interaction of the TCR complex (3) with antigen presented in the context of class II MHC molecules on the APC (4). This antigen-specific signal is not sufficient to generate a full response, and in the absence of a second signal may lead to clonal inactivation or anergy (5). The requirement for a second "costimulatory" signal provided by the APC has been demonstrated in a number of experimental systems (5, 6). The molecular nature of these second signal(s) is not completely understood, although both soluble molecules, such as IL-1 (6), and membrane-bound adhesion receptors (7) can provide costimulatory signals in some systems.

Studies with mAbs have indicated that the homodimeric T cell surface molecule CD28 may also provide a costimulatory signal to T lymphocytes (8). CD28 appears to function as a regulator of T cell-derived cytokines (9), primarily by stabilizing their mRNAs (10), but also by regulating transcription of these genes (8, 11). Earlier studies from our laboratory have shown that CD28 is a counter-receptor for the B cell activation antigen B7 (12). More recently, we have characterized the interactions between CD28 and B7 using genetic fusions of the extracellular portions of B7 and CD28, and Ig Cγ1 chains (13). Immobilized B7Ig fusion protein, as well as B7+ CHO cells, costimulated T cell proliferation. T cell stimulation with B7+ CHO cells also specifically stimulated increased levels of IL-2 transcripts. An indication of the role of CD28/B7 interactions in the functional collaboration between Th and B lymphoid cells was provided by the demonstration that mAbs to both CD28 and B7 specifically blocked Th-mediated Ig production by B cells (14). Additional studies by others have shown that anti-CD28 mAb inhibited IL-2 production induced in certain T cell leukemia cell lines by cellular interactions with a B cell leukemia line (15).

Structurally, CD28 is a member of the Ig superfamily, having a single extracellular V-like domain (16). A homologous molecule, CTLA-4, was identified by differential screening of a murine cytolytic T cell cDNA library (17).
Transcripts for this molecule were found in T cell populations having cytotoxic activity, suggesting that CTLA-4 might function in the cytolytic response (17, 18). A gene for the human counterpart of CTLA-4 has also been cloned and mapped (19) to the same chromosomal region (2q33-34) as CD28 (20). Sequence comparison between human CTLA-4 and CD28 proteins revealed significant homology between the proteins, with the greatest degree of homology being in the juxtamembrane and cytoplasmic regions (18, 19).

The high degree of homology between CD28 and CTLA-4, together with the colocalization of their genes, raises questions as to whether these molecules are also functionally related. However, since a protein product of CTLA-4 has not yet been identified, these questions remain unanswered. In this paper, we have examined the ability of CTLA-4 to bind the B7 counter-receptor. We have taken the approach of constructing a genetic fusion encoding CTLA4Ig, a soluble molecule comprising the extracellular domain of CTLA-4 joined to an Ig C1 chain. We have studied binding properties of CTLA 4Ig and have determined its functional effects on in vitro T and B lymphocyte responses.

Materials and Methods

mAbs and Ig Fusion Proteins. Murine mAbs 9.3 (anti-CD28) and G19-4 (anti-CD3), G3-7 (anti-CD7), BB-1 (anti-B7 antigen), and rat mAb 187.1 (anti-mouse κ chain) have been described previously (21-23), and were purified from ascites before use. The hybridoma producing mAb OKT8 was purchased from the American Type Culture Collection (Rockville, MD) and the mAb was also purified from ascites before use. mAb 4G9 (anti-CD19) was generously provided by Dr. E. Engleman (Stanford University). Purified human-mouse chimeric mAb L6 (having human Cγ1 Fc portion) was a gift of Margit Gayle and Dr. Perry Fell (Oncogen, Seattle, WA). Receptor Ig Cγ fusion proteins B7Ig, CD5Ig, and CD28Ig were prepared as previously described (13).

Cell Culture and Transfections. COS cells were transfected with expression plasmids as described (13). Ig fusion proteins were purified from serum-free conditioned medium from transfected COS cells by protein A affinity chromatography (13). B7+ CHO and CD28+ CHO have been previously described (13). CD7+ CHO cells were isolated by cosmid transfection and amplification of an expression CD7 cDNA clone (24) as described (12). CHO transfectants were maintained in DMEM supplemented with 10% FCS, 0.2 mM proline, and 1 μM methotrexate. The EBV-transformed lymphoblastoid cell lines (LCL),1 PM and TS1, were maintained in RPMI 1640 supplemented with 10% FCS.

Polymerase Chain Reaction. DNA fragments were amplified by PCR (25), using primer pairs described below. PCR reactions (0.1 ml final volume) were run in Taq polymerase buffer (Stratagene, Torrey Pines, CA), containing 20 μmol each dNTP; 50-100 pmol of the indicated primers; template (1 ng plasmid or cDNA synthesized from <1 μg total RNA using random hexamer primer; reference 26); and Taq polymerase (Stratagene). Reactions were run on a thermocycler (Perkin Elmer-Cetus) for 16-30 cycles (a typical cycle consisted of steps of 1 min at 94°C, 1-2 min at 50°C, and 1-3 min at 72°C).

Plasmid Construction. cDNAs encoding CD7 (24), CD28 (16), and B7 (27) have been described previously. Construction of OMCD28, a CD28 cDNA modified for more efficient expression by replacing the signal peptide with the analogous region from oncostatin M (28), and the B7Ig and CD28Ig expression plasmids have also been described (13).

A genetic fusion encoding CTLA4Ig construct was made essentially as described previously for the CD28Ig construct (13). The extracellular domain of CTLA-4 was cloned by PCR using synthetic oligonucleotides corresponding to the published sequence (19). Since a signal peptide for CTLA-4 was not identified in the CTLA-4 gene, the NH2 terminus of the predicted sequence of CTLA-4 was fused to the signal peptide of oncostatin M (28) in two steps using overlapping oligonucleotides. For the first step, the oligonucleotide TCTAGCTGTTCTGCACTCTGGTTTCCAAGCATGGCGAGCATGGCAATGCAGTGACGCCACGCC (which corresponded to the COOH-terminal 15 amino acids from the oncostatin M signal peptide fused to the NH2-terminal seven amino acids of CTLA-4) was used as forward primer, and TTGGGCTCTCTGATCAGAATCTGGGCACGGTTC (corresponding to residues 119-125 of CTLA-4 and containing a BclI restriction enzyme site) as reverse primer. The template for this step was cDNA synthesized from total RNA from H38 cells (an HTLV II-infected T cell leukaemic cell line kindly provided by Drs. Z. Salahu din and R. Gallo, National Cancer Institute). A portion of the PCR product from the first step was reassamlied, using an overlapping forward primer, corresponding to the NH2-terminal portion of the oncostatin M signal peptide and containing a HindIII restriction endonuclease site, CTAGCAGACTGAGCTTCCAACAAGGGTGTTACTGCTCACAAGAGACGTCTGCTAGCTGGTCCTTTGCACCTC and the same reverse primer. The product of this PCR reaction was digested with HindIII and BclI and ligated together with a BclI/XbaI-cleaved cDNA fragment encoding the hinge, CH2, and CH3 regions of Ig Cγ1 (13) into the HindIII/XbaI-cleaved expression vector CM8 (Invitrogen, San Diego, CA).

The sequence of the resulting CTLA4Ig fusion was determined and found to correspond exactly to the published sequence of CTLA-4, except that the codon for amino acid 111 was determined to be ACC rather than GCC as originally published (19); this difference was also confirmed on an independent clone spanning this region. The result of this difference is that the codon for amino acid 111 specifies threonine rather than alanine, as previously described.

To reconstruct full-length human CTLA-4, a cDNA fragment encoding the transmembrane and cytoplasmic domains was cloned by PCR and then joined with a fragment from CTLA4Ig encoding the oncostatin M signal peptide fused to the NH2 terminus of CTLA-4. For this purpose, the oligonucleotide GCATGCCAGCTGGCCAAGCCTGTGTTGCTACTG (corresponding to the first 11 amino acids in the predicted coding sequence) was used as a forward primer, and TGATGTAACATGTCTAGATCAATTGATGCTGGCCCAGCCTGCTGTGGTACTG (corresponding to residues 119-125 of CTLA-4 and containing a BclI restriction enzyme site) as reverse primer. Product was again cDNA synthesized from H38 cells. Products of the PCR reaction were cleaved with the restriction endonucleases Neol and XbaI, and the resulting 316-bp product was gel purified. A ~340-bp HindIII/Neol fragment from the CTLA4Ig fusion described above was also gel purified, and both restriction fragments were ligated into HindIII/XbaI cleaved CDM8. The resulting construct encoded full-length CTLA-4 and is designated OMCTLA4. CTLA-4 encoded by this construct also contained threonine at position 111, rather than alanine as reported previously (19).
Ligation products were transformed into MC1061/p3 cells and colonies were screened for the appropriate plasmids. Sequences of the resulting constructs were confirmed by DNA sequence analysis using a commercial kit (United States Biochemical Corp., Cleveland, Ohio).

**Immunostaining and FACS® Analysis.** Transfected cells or LCL were analyzed by indirect immunostaining. Before staining, COS and CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with mAbs or Ig fusion proteins at 10 µg/ml in DMEM containing 10% FCS for 1-2 h at 4°C. Cells were then washed and incubated for an additional 0.5-2 h at 4°C with FITC-conjugated goat anti-mouse Ig or with FITC-conjugated goat anti-human Ig Cγ serum (both from Tago Corp., Burlingame, CA). When binding of both mAbs and Ig fusion proteins was measured in the same experiment, FITC-conjugated anti-mouse and anti-human second step reagents were mixed together before use. Fluorescence on a total of 10,000 cells was then analyzed by FACS® (Becton Dickinson & Co., Mountain View, CA).

**PBL Separation and Stimulation.** PBL were isolated by centrifugation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD). Alloreactive T cells were isolated by stimulation of PBL in a primary MLR. PBL were cultured at 10⁶/ml in RPMI 1640 supplemented with 10% FCS in the presence of 2.5 × 10⁵/ml irradiated (5,000 rad) T51 LCL. After 6 d, alloreactive “blasts” were cryopreserved. Secondary MLR were conducted by culturing thawed alloreactive blasts together with fresh irradiated T51 LCL in the presence and absence of mAbs and Ig fusion proteins. Cells were cultured in 96-well flat-bottomed plates (4 × 10⁴ alloreactive blasts and 10⁴ irradiated T51 LCL cells/well) in a volume of 0.2 ml of RPMI 1640 containing 10% FCS. Cellular proliferation of quadruplicate cultures was measured by uptake of [³H]thymidine during the last 6 h of a 2-3-d culture.

CD4⁺ T cells were isolated from PBL as described (29). B cells were purified by positive selection from peripheral blood by panning (30) with anti-CD19 mAb 4G9. To measure Th-induced Ig production, 10⁶ CD4⁺ T cells were mixed with 10⁶ allogeneic CD19⁺ B cells in 1 ml of RPMI 1640 containing 10% FCS. After culture for 6 d at 37°C, production of human IgM was measured in the culture supernatants as described (14).

**Binding Assays.** B7Ig was labeled with ¹²⁵I to a specific activity of ~2 × 10⁶ cpm/nmol. 96-well plastic dishes were coated for 16–24 h with a solution containing CTLA4Ig (0.5 µg in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were blocked with binding buffer (DMEM containing 50 mM BES [Sigma Chemical Co.], pH 6.8, 0.1% BSA, and 10% FCS) before addition of a solution (0.09 ml) containing ¹²⁵I-B7Ig (~5 × 10⁶ cpm) in the presence or absence of competitor. After incubation for 2–3 h at 23°C, wells were washed once with binding buffer and four times with PBS. Bound radioactivity was then solubilized by addition of 0.5 N NaOH, and quantitated by gamma counting.

**Results**

**Construction of a Soluble CTLA-4 Ig Cγ Fusion Protein.** We previously have shown that CD28Ig, the soluble product of a genetic fusion between the extracellular domain of CD28 and an Ig Cγ 1 domain, bound the B7 counter-receptor (13). To determine whether CTLA-4 also bound B7, we constructed an analogous genetic fusion between the extracellular domain of CTLA-4 and an Ig Cγ 1 domain (Fig. 1A).

Since the expression of CTLA-4 in human lymphoid cells has not been reported, it was necessary to first find a source of CTLA-4 mRNA. We therefore screened by PCR cDNA made from total cellular RNA of different human leukemia cell lines using as primers oligonucleotides derived from the published sequence of the CTLA-4 gene (19). Of the cDNAs tested, H38 cells (an HTLV-1-associated leukemia line) gave the best yield of PCR products having the expected size. A cDNA containing the predicted coding sequence of the extracellular domain of CTLA-4 was then assembled from two PCR fragments amplified from H38 cDNA, as described in Materials and Methods. This fragment was digested with appropriate restriction enzymes and ligated together with a cDNA encoding the hinge, CH2, and CH3 domains of human Ig Cγ 1, into the mammalian expression vector, CDM8. Several isolates were transfected into COS cells, and supernatants were tested by indirect immunofluorescence and FACS® analysis for the presence of proteins that bound to B7⁺ CHO cells. Preliminary experiments indicated that several isolates encoded proteins that bound to B7⁺ CHO cells (see below). The DNA sequence of one such isolate was then determined and found to encode CTLA4Ig, as shown in Fig. 1A. CTLA4Ig contained a single base pair difference with the published sequence of CTLA-4 (19), such that an alanine residue at position 111 was changed to threonine. Threonine is also present at this position in the aligned sequences of murine CTLA-4 (17), murine CD28 (30), and human CD28 (16, 31).

CTLA4Ig was purified by protein A chromatography from serum-free culture supernatants of transfected COS cells (Fig. 1B). Under nonreducing conditions CTLA4Ig migrated as a Mr ~100,000 species, and as a Mr ~50,000 species under reducing conditions (Fig. 1B). CTLA4Ig therefore behaves during SDS-PAGE as a disulfide-linked dimer. Since the Ig Cγ hinge disulfides were eliminated during construction, a native disulfide linkage in CTLA-4 is most likely involved in this bond formation. CD28Ig also behaves as a disulfide-linked dimer during SDS-PAGE (13).

**Binding Activity of CTLA4Ig.** The binding activities of purified CTLA4Ig and CD28Ig (13) on B7⁺ CHO cells and on a lymphoblastoid cell line (PM LCL) were compared in the experiment shown in Fig. 2. Transfected CHO cell lines and PM LCL were incubated with equivalent concentrations (10 µg/ml) of CD51g, CD28Ig, or CTLA4Ig, and binding was detected by FACS® after addition of an FITC-conjugated second-step reagent. CD28Ig bound weakly but significantly to B7⁺ CHO cells and not at all to PM LCL. The B7⁺ CHO cells used in this experiment expressed lower amounts of B7 than those used in previous experiments (13) and bound correspondingly lower levels of CD28Ig. CTLA4Ig bound more strongly to both cell lines than CD28Ig. The difference between CD28Ig and CTLA4Ig binding to B7⁺ CHO cells was maintained when concentrations of the fusion proteins were titrated (data not shown). Neither CD28Ig nor CTLA4Ig bound to control CD28⁺ CHO cells.

To test whether CTLA-4 expressed on the cell surface membrane bound the B7 antigen, we cloned by PCR a cDNA (OMCTLA4) encoding full-length mature human CTLA-4 fused to the oncostatin M signal peptide (see Materials and
Figure 1. Construction and expression of CTLA4Ig. (A) Map of CTLA4Ig constructs. A cDNA construct encoding the indicated portions of oncostatin M (dark shaded regions), CTLA-4 (unshaded regions), and human Ig Cy1 (stippled regions) was constructed as described in Materials and Methods. Sequences displayed show the junctions between CTLA-4 (capital letters), and the signal peptide (SP) of oncostatin M, and the hinge (H) of Ig Cy1. The amino acid in parentheses was introduced during construction. Asterisks denote cysteine to serine mutations introduced in the Ig Cy hinge region. The Ig superfamily V-like domain present in CTLA-4 is indicated, as are the CH2 and CH3 domains of Ig Cy1. (B) Purification of CTLA4Ig. An expression plasmid construct encoding CTLA4Ig was transfected into COS cells, and Ig Cy-containing proteins were purified from serum-free-conditioned medium. Concentrations of CTLA4Ig were determined assuming an extinction coefficient at 280 nm of 1.6 (experimentally determined by amino acid analysis of a solution of known absorbance). Molecular weight standards (lanes 1 and 3) and samples (1 μg) of CTLA4Ig (lanes 2 and 4) were subjected to SDS-PAGE (4–12% acrylamide gradient) under nonreducing (-βME, lanes 1 and 2) or reducing (+βME, lanes 3 and 4) conditions. Proteins were visualized by staining with Coomassie brilliant blue.

Methods). COS cells were transfected with expression plasmids CD7, OMCD28, and OMCTLA4, and 48 h later, cells were tested for expression of the appropriate cell surface markers by FACS® analysis (data not shown). The B7lg fusion protein (but not control CD5lg fusion protein) bound to both CD28- and CTLA-4 transfected cells. mAb 9.3 bound to CD28-transfected COS cells, but not to CTLA-4-transfected cells. CD7-transfected COS cells bound neither mAb 9.3 nor either of the fusion proteins. Thus, membrane-bound CTLA-4 and soluble CTLA4 Ig both bound the B7 counter-receptor.

We measured the apparent avidity of interaction between CTLA4Ig and B7lg using a solid phase binding assay. B7lg was radiolabeled with 125I, and bound to immobilized CTLA4Ig in the presence or absence of increasing concentrations of various competitors (Fig. 3 A). Only anti-B7 mAb BB-1 and unlabeled B7lg competed significantly for 125I-B7lg binding (half-maximal effects at ~175 and ~22 nM, respectively). The slope of the inhibition curve for mAb BB-1 was different than the slope of the curve for B7lg, and does not appear to reach the same maximal inhibition. The inefficiency of mAb BB-1 (an IgM mAb) at 125I-B7lg binding competition was also observed in other experiments and may indicate that the mAb has lower affinity for B7lg or that the BB-1 epitope does not exactly coincide with the binding site for CTLA4Ig. Neither chimeric mAb L6 (a nonbinding control Ig molecule), nor anti-CD28 mAb 9.3 competed effectively, although the highest concentration of mAb 9.3 used was sufficient to inhibit binding 125I-B7lg to immobilized CD28lg or to cell surface expressed CD28 by ≥90% (13). The competition data from Fig. 3 A were plotted in the Scatchard representation, and a $K_d \sim 12$ nM was calculated for binding of 125I-B7 to immobilized CTLA4lg (Fig. 3 B).

Immunoprecipitation Analysis of Cell Surface Proteins Binding CTLA4Ig. We next compared by immunoprecipitation analysis the abilities of CD28lg, CTLA4lg, and CD5lg to bind solubilized B7 from 125I-surface labeled cells. As shown in Fig. 4, a diffusely migrating (Mr ~50,000–75,000; center at ~60,000) radiolabeled protein was immunoprecipitated by
Figure 3. $^{125}$I-labeled B7lg binds with high avidity to immobilized CTLA4lg. (A) Competition binding analysis of $^{125}$I-B7lg. 96-well plastic dishes were coated with CTLA4lg as described in Materials and Methods. $^{125}$I-labeled B7lg (5 x $10^5$ cpm, 2 x $10^6$ cpm/pmol) was then added to a concentration of 4 nM in the presence of the indicated concentrations of unlabeled chimeric mAb L6, mAb 9.3, mAb BB-1, or B7lg. Plate-bound radioactivity was determined and is expressed as a percentage of radioactivity bound to wells treated without competitor (28,300 cpm). Each point represents the mean of triplicate determinations; replicates varied from the mean by $\pm 20\%$. Concentrations were calculated based on a $M_r$ of 75,000 per binding site for mAbs and 51,000 per binding site for B7lg. (B) Scatchard analysis of $^{125}$I-lg binding to immobilized CTLA4lg. Data from the unlabeled B7lg competition binding experiment shown in A were replotted in the Scatchard representation and a binding constant ($K_d$) was estimated from the slope of the line best fitting the experimental data ($r = -0.963$).

CTLA4lg Is a Potent Inhibitor of Immune Responses In Vitro Previous studies showed that anti-CD28 mAb, 9.3, and anti-B7 mAb, BB-1, inhibited proliferation of alloantigen specific T cells, as well as Ig secretion by alloantigen-presenting B cells (14, 32-34). These observations suggested that soluble forms of CD28, CTLA-4, or B7 might also inhibit these responses. The effects of CD28lg, CTLA4lg, and B7lg on T cell proliferation in an MLR were compared in the experiment shown in Fig. 5. CTLA4lg inhibited the MLR reaction in a dose-dependent fashion by a maximum of 790%, with half-maximal response at $\sim 30$ ng/ml ($\sim 0.8$ nM). The Fab fragment of mAb 9.3, a more potent inhibitor of MLR than whole mAb 9.3 (32), was also inhibitory, but at much higher concentrations ($\sim 800$ ng/ml or $\sim 30$ nM for half-maximal response). B7lg and CD28lg did not significantly inhibit the MLR even at higher concentrations. In other experiments, addition of B7lg together with CTLA4lg reversed the inhibition of MLR by CTLA4lg, indicating that the inhibition was specifically due to interactions with B7 (data not shown).

The effects of CTLA4lg on Th induced Ig secretion was also examined (Fig. 6). CD4$^+$ T cells stimulated IgM production by allogeneic CD19$^+$ B cells. In the absence of CD4$^+$ T cells, IgM levels were only $\sim 7\%$ of levels measured in the presence of CD4$^+$ T cells. As shown previously (14), mAbs 9.3 and BB-1 significantly inhibited Th-induced IgM production (63% and 65% inhibition, respectively). CTLA4lg was even more effective as an inhibitor (89% inhibition) than were these mAbs. Inhibition by control Ig molecules, mAbs OKT8 and CD5lg, was much less. None of
these molecules significantly inhibited Ig production measured in the presence of *Staphylococcus aureus* enterotoxin B (data not shown). Similar results were obtained with CD4+ T cells and B cells derived from other donors.

**Discussion**

Previous studies showed that CTLA-4 and CD28 form a subgroup of closely related molecules belonging to the Ig superfamily (35). Here we have shown that CTLA-4 and CD28 are functionally as well as structurally related. Soluble CTLA4Ig bound specifically to the surface of B7+ CHO cells and to a LCL (Fig. 2). CTLA4Ig also specifically immunoprecipitated identically sized proteins from extracts of these cells (Fig. 4), indicating that CTLA4Ig retained binding activity for solubilized B7. Finally, the Kd of binding of soluble 125I-B7lg for immobilized CTLA4Ig was estimated as ~12 nM (Fig. 3 B). This apparent Kd compares favorably with higher affinity mAbs (Kd 2-10,000 nM; reference 36) and is similar to or greater than Kd values of integrin receptors and their ligands (10-2,000 nM; references 37-39); the binding of soluble alloantigen to the TCR of a murine T cell hybridoma (~100 nM; reference 40); interactions between CD2 and LFA3 (400 nM; reference 41); and interactions between CD4 and MHC class II molecules (42). Taken together, these observations indicate that CTLA-4 is a receptor for the B cell activation antigen, B7. Thus, both CD28 and CTLA-4 bind the same counter-receptor.

Side-by-side comparisons were made of the abilities of CD28Ig and CTLA4Ig to directly bind to membrane bound B7 (Fig. 2), to immunoprecipitate B7 solubilized from different cell types (Fig. 4), and to inhibit T cell proliferation in an MLR (Fig. 5). In all cases, CTLA4Ig was more effective than CD28Ig. We have also found that CTLA4Ig is a much more effective competitor for binding of biotinylated CTLA4Ig to B7+ CHO cells than is CD28Ig (data not shown). The avidity of soluble 125I-labeled B7lg for immobilized CTLA4Ig (Kd ~12 nM) was also found to be ~20-fold greater than its avidity under identical conditions for immobilized CD28Ig (Kd ~200 nM; reference 13). A preponderance of evidence suggests, therefore, that CTLA4Ig has higher avidity for B7 than does CD28Ig.

There are several possible explanations for this finding. First, it is possible that the Ig fusion proteins do not accurately reflect native interactions between membrane-bound CTLA-4, CD28, and the B7 counter-receptor. Perhaps the low relative avidity of CD28Ig indicates that this protein does not retain full binding activity. We feel this possibility is unlikely since in previous studies the avidities of 125I-B7lg for immobilized CD28Ig and CD28Ig expressed on the membrane of transfected cells were found to be identical (13). We did not measure avidity between 125I-B7lg and membrane-bound CTLA-4 in the present study, so we do not know if CTLA4Ig likewise retains most of the binding activity of membrane-bound CTLA-4. However, the high Kd of interaction between CTLA4Ig and B7lg argues in favor of this being the case.

It is also possible that the high apparent Kd of CTLA4Ig is the result of greater valency of this fusion protein. CTLA4Ig forms a disulphide-linked dimer of Mr 50,000 subunits (Fig. 1 B). Since no interchain disulfides would be predicted to form in the Ig portion of this fusion, it seems likely that
cysteines from CTLA-4 are involved in disulfide bond forma-
tion. The analogous CD28Ig fusion protein (13) also con-
tains interchain disulfide linkage(s). The sizes in solution of
CTLA4Ig and CD28Ig were also compared by size fractiona-
ton on a TSK G3000SW column eluted with PBS. CTLA4Ig
eluted with an apparent $M_r$ of $\sim 200,000$, while CD28Ig
eluted with an apparent $M_r$ of $\sim 300,000$. Both CTLA4Ig
and CD28Ig behave in solution as molecules approximately
twice as large as their apparent $M_r$s determined by SDS-
PAGE (reference 13; and Fig. 2), suggesting that they form
higher aggregates, possibly tetramers. It is not known how
many binding sites for B7 these molecules contain, but the
difference in apparent avidities of CD28Ig and CTLA4Ig is
not easily explainable by differences in their size or degree
of aggregation in solution.

Finally, it is possible that the greater apparent avidity of
CTLA4Ig indicates that CTLA-4 is a higher avidity receptor
for B7. Proof of this point will require studies comparing the
affinities of membrane-bound CD28 and CTLA-4 for soluble
and membrane-bound B7. These studies are technically
difficult at the present time. The OMC: CTLA4 cDNA
clone is expressed poorly in COS and CHO cells (Linsley,
P.S., unpublished observations), making binding measure-
tments to CTLA-4-transfected cells more difficult. Likewise,
since we previously showed by binding inhibition and im-
munoprecipitation experiments that CD28 was the major B7Ig
binding protein from activated PBL (13), the levels of ex-
pression of native CTLA-4 in activated PBL are likely to be
less than levels of CD28. This is consistent with preliminary
results from RNA blotting experiments showing that
CTLA-4 transcripts are more generally present at lower levels in cell
lines and in PBL than CD28 transcripts (Urnes, M., and P.S.
Linsley, unpublished observation). These findings may indi-
cate that CTLA-4 is a receptor of low abundance, but high
avidity.

Functional consequences of CTLA-4 binding to B7 are not
currently known, although if CTLA-4 has signalling prop-
erties, these are likely mediated by its cytoplasmic domain.
The cytoplasmic domains of murine and human CTLA-4 are
identical (19), suggesting that this region has important func-
tional properties. The cytoplasmic domains of CD28 and
CTLA-4 also share significant homology (Linsley, P.S., un-
published observation). Evaluation of signalling by CTLA-4
will require development of reagents that specifically trigger
this molecule. It is possible that B7Ig (13) could be used to
study CTLA-4 signalling, although these studies would be
complicated by the fact that B7 binds both CD28 and CTLA-4.
How each of these receptors contributes to overall signalling
mediated by B7 will depend upon their relative avidities for
B7, as well as their levels of expression.

CTLA4Ig is a potent inhibitor of in vitro lymphocyte func-
tions requiring T cell and B cell collaboration (data not shown).
These findings, together with previous studies (14), indicate the
fundamental importance of interactions between B7 and
its counter-receptors, CD28 and/or CTLA-4, in regulating
both T and B lymphocyte responses. CTLA4Ig should be
useful for future investigations on the role of these interac-
tions during immune responses. CTLA-4 is a more potent
inhibitor of in vitro lymphocyte responses than either mAb
BB-1 or mAb 9.3 (data not shown). The greater potency of
CTLA4Ig over mAb BB-1 is most likely due to the differ-
ence in affinities for B7 between these molecules (Fig. 3 A).
CTLA4Ig is also more potent than Fab fragments of mAb
9.3, which are more inhibitory than whole mAb 9.3 (32).
The immunosuppressive effects of CTLA4Ig in vitro sug-
Suggest possible therapeutic uses of this molecule for treatment
of autoimmune disorders involving aberrant T cell activa-
tion or Ig production.

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