CTLA-4 Is a Second Receptor for the B Cell Activation Antigen B7

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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 Cγ 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 ^{125}I-labeled extracts of these cells. The avidity of ^{125}I-labeled B7Ig fusion protein for immobilized CTLA4Ig was estimated (K_d ~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.

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).

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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 Cγ1 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 cotransfection and amplification of an expressible 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 T51, 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 NH_2 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 CTCAGTCTGGTCCTTGCACTCCTGTTTCCAAGCATGGCGAGCATGGCAATGCACGTGGCCCAGCC (which corresponded to the COOH-terminal 15 amino acids from the oncostatin M signal peptide fused to the NH_2-terminal seven amino acids of CTLA-4) was used as forward primer, and TTTGGGCTCCTGATCAGAATCTGGGCACGGTTC (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 leukemic cell line kindly provided by Drs. Z. Salahudin and R. Gallo, National Cancer Institute). A portion of the PCR product from the first step was reamplified, using an overlapping forward primer, corresponding to the NH_2-terminal portion of the oncostatin M signal peptide and containing a HindIII restriction endonuclease site, CTAGCCACTGAAGCTTCACCAATGGGTGTACTGCTCACACAGAGGACGCTGCTCAGTCTGGTCCTTGCACTC 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 CDM8 (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 NH_2 terminus of CTLA-4. For this purpose, the oligonucleotide GCAATGCACGTGGCCCAGCCTGCTGTGGTACTG (corresponding to the first 11 amino acids in the predicted coding sequence) was used as a forward primer, and TGATGTAACATGTCTAGATCAATTGATGGGAATAAAATAAGGCTG (corresponding to the last eight amino acids in CTLA-4 and containing an XbaI site) as reverse primer. Template again was cDNA synthesized from H38 cells. Products of the PCR reaction were cleaved with the restriction endonucleases NcoI and XbaI, and the resulting 316-bp product was gel purified. A ~340-bp HindIII/NcoI fragment from the CTLAIg fusion described above was also gel purified, and both restriction fragments were ligated into HindIII/Xba-I 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).

[1] *Abbreviation used in this paper:* LCL, lymphoblastoid cell lines.

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 10^6/ml in RPMI 1640 supplemented with 10% FCS in the presence of 0.25 × 10^6/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 (4×10^4 alloreactive blasts and 10^4 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 [^{3}H]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, 10^6 $CD4^+$ T cells were mixed with 10^6 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 ^{125}I to a specific activity of ~2 × 10^6 cpm/pmol. 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 ^{125}I-B7Ig (~5 × 10^5 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 quantified 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 Cγ1 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 Cγ1 domain (Fig. 1 *A*).

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 II–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 Cγ1, 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. 1 *A*. 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. 1 *B*). Under nonreducing conditions CTLA4Ig migrated as a M_r ~100,000 species, and as a M_r ~50,000 species under reducing conditions (Fig. 1 *B*). 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 CD5Ig, 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

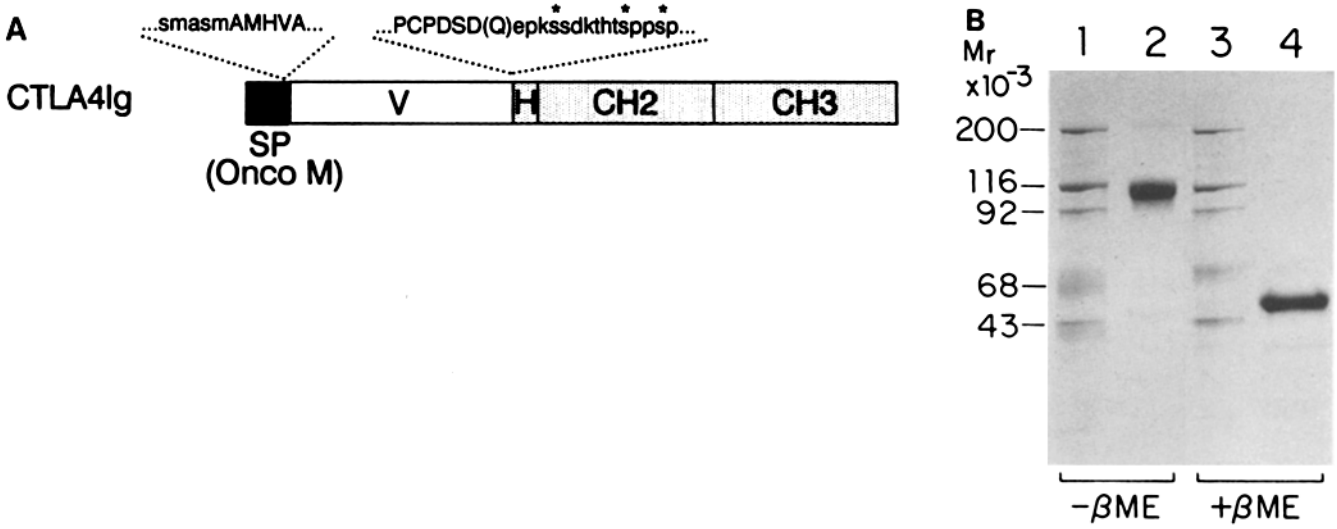


Figure 1. Construction and expression of CTLA4Ig. (*A*) Map of CTLA4Ig constructs. A cDNA construct encoding the indicated portions of oncostatin M (*dark shaded regions*), CTLA-4 (*unshaded regions*), and human Ig Cγ1 (*stippled regions*) was constructed as described in Materials and Methods. Sequences displayed show the junctions between CTLA-4 (*capital letters*), and the signal peptide (SP) of oncostatin M, and the hinge (H) of Ig Cγ1. The amino acid in parentheses was introduced during construction. Asterisks denote cysteine to serine mutations introduced in the Ig Cγ hinge region. The Ig superfamily V-like domain present in CTLA-4 is indicated, as are the CH2 and CH3 domains of Ig Cγ1. (*B*) Purification of CTLA4Ig. An expression plasmid construct encoding CTLA4Ig was transfected into COS cells, and Ig Cγ-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 B7Ig fusion protein (but not control CD5Ig 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.

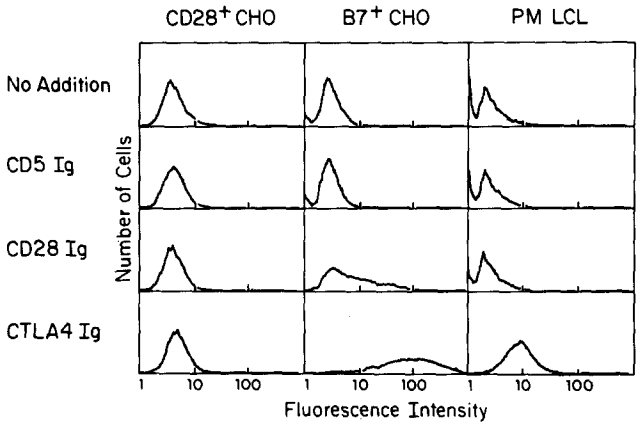
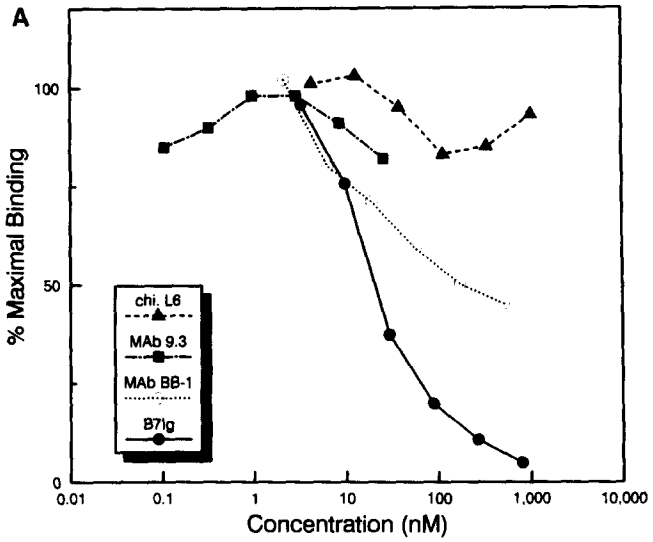


Figure 2. CTLA4Ig binds specifically to cells that express the B7 antigen. Amplified transfected CHO cells expressing CD28 or B7, and PM LCL cells were incubated with medium only (no addition), or human Ig Cγ1-containing proteins CD5Ig, CD28Ig, or CTLA4Ig, followed by FITC-conjugated goat anti-human Ig second-step reagents. A total of 10,000 stained cells was then analyzed by FACS®.

We measured the apparent avidity of interaction between CTLA4Ig and B7Ig using a solid phase binding assay. B7Ig was radiolabeled with ^{125}I, 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 B7Ig competed significantly for ^{125}I-B7Ig 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 B7Ig, and does not appear to reach the same maximal inhibition. The inefficiency of mAb BB-1 (an IgM mAb) at ^{125}I-B7Ig binding competition was also observed in other experiments and may indicate that the mAb has lower affinity for B7Ig 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 ^{125}I-B7Ig to immobilized CD28Ig 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 ~12 nM was calculated for binding of ^{125}I-B7 to immobilized CTLA4Ig (Fig. 3 *B*).

Immunoprecipitation Analysis of Cell Surface Proteins Binding CTLA4Ig. We next compared by immunoprecipitation analysis the abilities of CD28Ig, CTLA4Ig, and CD5Ig to bind solubilized B7 from ^{125}I-surface labeled cells. As shown in Fig. 4, a diffusely migrating (M_r ~50,000–75,000; center at ~60,000) radiolabeled protein was immunoprecipitated by



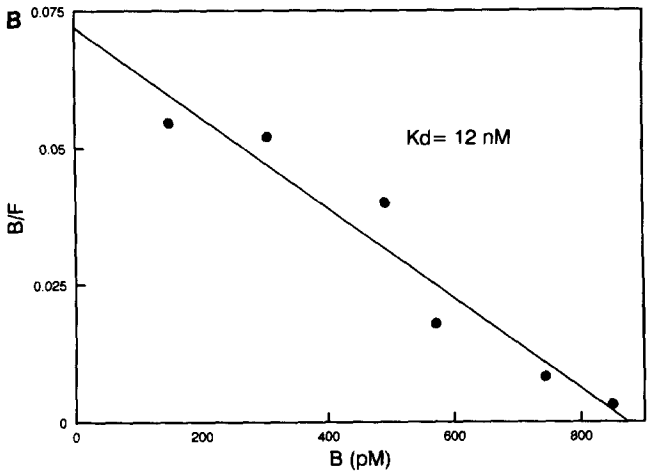


Figure 3. ^{125}I-labeled B7Ig binds with high avidity to immobilized CTLA4IG. (*A*) Competition binding analysis of ^{125}I-B7Ig. 96-well plastic dishes were coated with CTLA4Ig as described in Materials and Methods. ^{125}I-labeled B7Ig (5×10^5 cpm, 2×10^6 cpm/pmol) was then added to a concentration of 4 nM in the presence of the indicated concentrations of unlabeled chimeric mAb L6, mAb 9.3, mAb BB-1, or B7Ig. 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 ≤20%. Concentrations were calculated based on a M_r of 75,000 per binding site for mAbs and 51,000 per binding site for B7Ig. (*B*) Scatchard analysis of ^{125}Ig binding to immobilized CTLA4Ig. Data from the unlabeled B7Ig 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 ($r = -0.963$).

CTLA4Ig from B7$^+$ CHO cell extracts. On longer exposure, a protein of this size was also visible in samples immunoprecipitated with CD28Ig, but not CD5Ig. In another experiment, this protein was again precipitated by CTLA4Ig from B7$^+$ CHO cells, but not from surface-labeled ^{125}I-labeled CD28$^+$ CHO cells (data not shown). CTLA4Ig, but not CD28Ig or CD5Ig, also immunoprecipitated a molecule of the same size from extracts of ^{125}I-labeled lymphoblastoid cells.

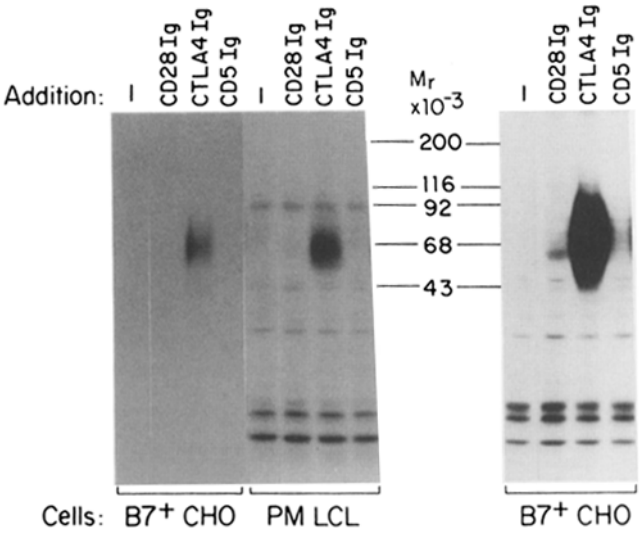


Figure 4. Immunoprecipitation of B7 from transfected CHO and lymphoblastoid cell lines by CTLA4Ig. B7$^+$ CHO and PM lymphoblastoid cells were surface labeled with ^{125}I, and extracted with a nonionic detergent solution (13). Aliquots containing ~1.5×10^7 cpm were subjected to immunoprecipitation analysis (13) with no addition, or 2 μg each of CD28Ig, CTLA4Ig, or CD5Ig. Washed immunoprecipitates were then analyzed by SDS-PAGE (10–20% acrylamide) under reducing conditions. The gel was then dried and exposed to X-ray film for 1 d (*left*) or 10 d (*center* and *right*).

CTLA4Ig 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 Th 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 CD28Ig, CTLA4Ig, and B7Ig on T cell proliferation in an MLR were compared in the experiment shown in Fig. 5. CTLA4Ig inhibited the MLR reaction in a dose-dependent fashion by a maximum of >90%, with half-maximal response at ~30 ng/ml (~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 (~800 ng/ml or ~30 nM for half-maximal response). B7Ig and CD28Ig did not significantly inhibit the MLR even at higher concentrations. In other experiments, addition of B7Ig together with CTLA4Ig reversed the inhibition of MLR by CTLA4Ig, indicating that the inhibition was specifically due to interactions with B7 (data not shown).

The effects of CTLA4Ig 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 ~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). CTLA4Ig was even more effective as an inhibitor (89% inhibition) than were these mAbs. Inhibition by control Ig molecules, mAbs OKT8 and CD5Ig, was much less. None of

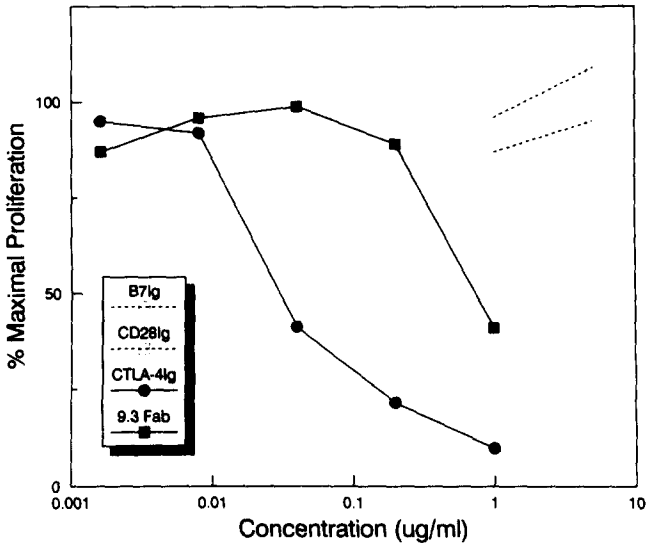


Figure 5. CTLA4Ig inhibits T cell proliferation in a mixed lymphocyte reaction. Primary MLR blasts were stimulated with irradiated T51 LCL in the absence or presence of the indicated concentrations of murine mAb 9.3 Fab fragments, or B7Ig, CD28Ig, or CTLA4Ig Ig Cγ fusion proteins. Cellular proliferation was measured by [^{3}H]thymidine incorporation after 4 d and is expressed as the percentage of incorporation by untreated cultures (21,000 cpm). Shown are the means of quadruplicate determinations (SEM ≤10%).

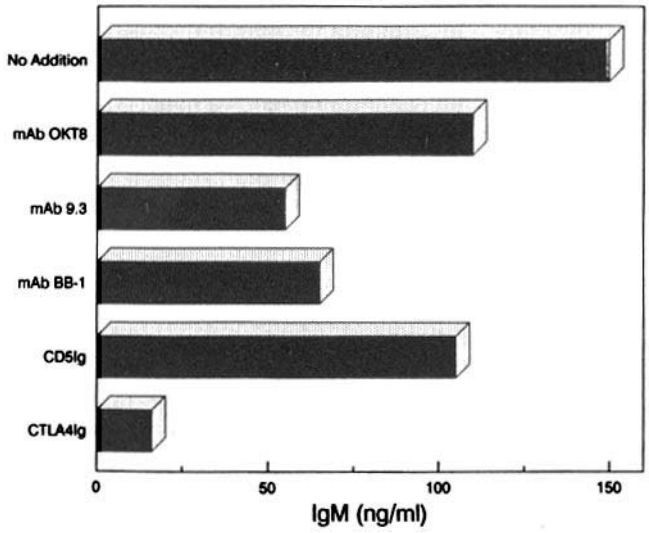


Figure 6. CTLA4Ig inhibits helper T cell–induced Ig production by human B cells. CD4$^+$ T cells were mixed with allogeneic CD19$^+$ B cells in the presence or absence of the indicated Ig molecules as described in Materials and Methods. Murine mAbs OKT8, 9.3, and BB-1 were added at 20 μg/ml, and Ig fusion proteins, at 10 μg/ml. After 6 d of culture, concentrations of human IgM (SEM <5%) in culture supernatants were determined as described (14). IgM production by B cells cultured in the absence of CD4$^+$ T cells was 11 ng/ml.

these molecules significantly inhibited Ig production measured in the presence of *Staphylococcus aureus* entorotoxin 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 K_d of binding of soluble ^{125}I-B7Ig for immobilized CTLA4Ig was estimated as ~12 nM (Fig. 3 *B*). This apparent K_d compares favorably with higher affinity mAbs (K_d 2–10,000 nM; reference 36) and is similar to or greater than K_d 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, CTL4Ig was more effective than CD28Ig. We have also found that CTL4Ig 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 ^{125}I-labeled B7Ig for immobilized CTLA4Ig (K_d ~12 nM) was also found to be ~20-fold greater than its avidity under identical conditions for immobilized CD28Ig (K_d ~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 ^{125}I-B7Ig for immobilized CD28Ig and CD28Ig expressed on the membrane of transfected cells were found to be identical (13). We did not measure avidity between ^{125}I-B7Ig 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 K_d of interaction between CTLA4Ig and B7Ig argues in favor of this being the case.

It is also possible that the high apparent K_d of CTLA4Ig is the result of greater valency of this fusion protein. CTLA4Ig forms a disulfide-linked dimer of M_r ~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 formation. The analogous CD28Ig fusion protein (13) also contains interchain disulfide linkage(s). The sizes in solution of CTLA4Ig and CD28Ig were also compared by size fractionation on a TSK G3000SW column eluted with PBS. CTLA4Ig eluted with an apparent M_r of ~200,000, while CD28Ig eluted with an apparent M_r of ~300,000. Both CTLA4Ig and CD28Ig behave in solution as molecules approximately twice as large as their apparent M_rs 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 OMCTLA4 cDNA clone is expressed poorly in COS and CHO cells (Linsley, P.S., unpublished observations), making binding measurements to CTLA-4-transfected cells more difficult. Likewise, since we previously showed by binding inhibition and immunoprecipitation experiments that CD28 was the major B7Ig binding protein from activated PBL (13), the levels of expression 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 indicate 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 properties, 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 functional properties. The cytoplasmic domains of CD28 and CTLA-4 also share significant homology (Linsley, P.S., unpublished 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 functions 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 interactions 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 difference 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 suggest possible therapeutic uses of this molecule for treatment of autoimmune disorders involving aberrant T cell activation or Ig production.

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