

# Coexpression and Functional Cooperation of CTLA-4 and CD28 on Activated T Lymphocytes

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## Summary

T cell costimulation by molecules on the antigen presenting cell (APC) is required for optimal T cell proliferation. The B7 molecule on APC binds the T lymphocyte receptor CD28, triggering increased interleukin 2 (IL-2) production and subsequent T cell proliferation. CTLA-4 is a predicted T cell membrane receptor homologous to CD28, which also binds the B7 counter receptor, but whose distribution and function are unknown. Here we have developed monoclonal antibodies (mAbs) specific for CTLA-4 and have investigated these questions. mAbs were produced that bound CTLA-4 but not CD28, and that blocked binding of CTLA-4 to B7. CTLA-4 expression as measured by these mAbs was virtually undetectable on resting T cells, but was increased several hundred-fold during T cell activation. On activated lymphocytes, CTLA-4 was expressed equally on CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets and was coexpressed with CD25, CD28, and CD45RO. CTLA-4 expression was lower than that of CD28, reaching a maximum of ~1/30–50 the level of CD28. Despite its lower expression, CTLA-4 was responsible for much of the B7 binding by large activated T cells. Anti-CTLA-4 mAb 11D4 and anti-CD28 mAb 9.3 acted cooperatively to inhibit T cell adhesion to B7, and to block T cell proliferation in primary mixed lymphocyte culture. When coimmobilized with anti T cell receptor (TCR) mAb, anti-CTLA-4 mAbs were less effective than anti-CD28 mAb 9.3 at costimulating proliferation of resting or activated T cells. However, coimmobilized combinations of anti-CD28 and anti-CTLA-4 were synergistic in their ability to augment anti-TCR-induced proliferation of preactivated CD4<sup>+</sup> T cells. These results indicate that CTLA-4 is coexpressed with CD28 on activated T lymphocytes and cooperatively regulates T cell adhesion and activation by B7.

T lymphocyte activation requires occupancy of the TCR by antigenic peptides in the context of MHC molecules on the APC. Other T cell surface molecules interact with specific counter receptors on the APC to receive costimulatory signals that regulate the outcome of TCR occupancy (1, 2). T cell activation in vitro in the absence of costimulation results in development of antigen-specific unresponsiveness by clonal anergy (2, 3), or activation-induced cell death (4). The molecular basis for T cell costimulation is not clearly understood, but may involve several different T cell surface receptors and their counter receptors on the APC (5).

CD28 is a T cell surface receptor that delivers important costimulatory signals. CD28 mediates a T cell activation pathway that has been extensively studied using mAbs (6). These studies have led to the conclusion that CD28 is an important regulator of T cell cytokine production by transcriptional (7, 8) and posttranscriptional (9) mechanisms. More recent studies have suggested a role for CD28 in regulation of clonal anergy. mAbs to CD28 augmented T cell prolifera-

tion in the presence of costimulation-deficient APC (10), and reversed development of clonal anergy in T cell clones (11). A counter receptor for CD28 is B7 (12), a molecule first identified as a B lymphocyte activation antigen (13). B7 has been shown to costimulate proliferation of human (14–17) and murine T cells (18, 19).

CTLA-4 is a predicted T cell surface molecule that was identified by differential screening of a murine cytolytic-T cell cDNA library (20). CTLA-4 transcripts were found in T cell populations having cytotoxic activity, leading to the suggestion that CTLA-4 might function in the cytolytic response (20, 21). CTLA-4 bears structural homology to CD28 (22, 23), and genes for these molecules colocalize to the same chromosomal regions in human (24) and mouse (23, 25). Like CD28, CTLA-4 also binds the B7 counter receptor (26). CTLA4Ig, an immunoglobulin Fc fusion of the extracellular domain of CTLA-4, bound B7 with an avidity ~20-fold higher than an analogous CD28Ig fusion protein, and blocked alloantigen responses in vitro (26). CTLA4Ig administration

in vivo suppresses antibody responses (27) and transplant rejection (28).

The normal function of CTLA-4 is unknown. It is unclear why distinct counter receptors for B7 have evolved. Until now, a protein product of the CTLA-4 gene has not been identified, and its cellular distribution and role in normal T cell immune responses are unknown. To address these problems, we have prepared mAbs specific for CTLA-4. In this study, we have used these reagents to address the distribution and function of CTLA-4 during T cell activation.

## Materials and Methods

**mAbs.** Murine mAbs 9.6 (anti-CD2), G19-4 (anti-CD3), G17-2 and 66.1 (anti-CD4), G10.1 (anti-CD5), 9.3 (anti-CD28), and rat mAb 187.1 (antimurine Ig  $\kappa$  chain) have been described previously (29–32). mAb 4G7 (anti-CD19) was a gift of E. Engleman (Stanford University, Stanford, CA) and UCHL1 (anti-CD45RO) was from P. Beverly (University College, London, UK). mAb WT31 (anti-TCR) was purchased from Becton Dickinson & Co. (San Jose, CA) and mAb M23 (antibreast tumor mucin) was acquired from Genetic Systems Corp. (Seattle, WA). mAb 7G7/B6 (anti-CD25) was obtained from the American Type Culture Collection (Rockville, MD).

**Cell Culture.** B7<sup>+</sup> CHO cells have been previously described (14, 26) and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS)<sup>1</sup>, 0.2 mM proline, and 1  $\mu$ M methotrexate. COS cells were maintained in DMEM supplemented with 10% FBS.

T cells were cultured in lymphocyte culture medium (RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin). PBL were isolated by centrifugation through lymphocyte separation medium (LSM; Organon Teknika Corp., Durham, NC). PHA-activated T cells were prepared by culturing PBL with 1  $\mu$ g/ml PHA (Wellcome Diagnostic, Charlotte, NC) in lymphocyte culture medium for 6 d, then in lymphocyte culture medium lacking PHA for 1 d. Viable cells were collected by sedimentation through LSM before use. Enriched CD4<sup>+</sup> or CD8<sup>+</sup> populations were isolated by complement-mediated lysis of CD8<sup>+</sup> or CD4<sup>+</sup> T cells (labeled with mAbs G10.1 or 66.1, respectively) before PHA activation. The resulting CD4<sup>+</sup> culture was ~90% CD4<sup>+</sup>, 2% CD8<sup>+</sup>; and the CD8<sup>+</sup> culture, ~95% CD8<sup>+</sup> and 1% CD4<sup>+</sup>, as judged by flow cytometry. In some experiments, PBL were also activated by immobilized anti-CD3 mAb G19-4. Plastic wells were coated with a solution of mAb G19-4 at 20  $\mu$ g/ml in PBS for ~2 h at 37°C and washed with PBS before addition of PBL.

CD4<sup>+</sup> T cells were isolated from PBL by immunomagnetic negative selection (33) and cultured in lymphocyte culture medium. The selection procedure routinely yielded preparations of >95% CD4<sup>+</sup> cells. Alloantigen-primed CD4<sup>+</sup> T cells were generated from MLC of CD4<sup>+</sup> T cells with an irradiated (10,000 rad) EBV-transformed lymphoblastoid cell line from an unrelated donor (DR2/DR4). At weekly intervals, cells were refed with fresh irradiated lymphoblastoid cells and IL-2 (20 U/ml) as previously described (33). Viable cells were isolated before use by sedimentation

through LSM. In some experiments, viable cells were rested for ~16–24 h in lymphocyte culture medium before use. Alloantigen-primed cells were cultured for 3–7 wk with weekly restimulation. CD4<sup>+</sup> T cells were also activated by coimmobilized anti-TCR mAb WT31 with intracellular adhesion molecule 1 receptor globulin (ICAM-1 Rg) fusion protein (33). Round bottomed 96-well microtiter plates were coated with goat anti-mouse and anti-human Ig, followed by mAb WT31 and ICAM Rg at 25 ng per well (33). CD4<sup>+</sup> T cells were added ( $5 \times 10^4$  per well) and cultured for 3 d at 37°C.

**Plasmids and Transfections.** Expression plasmids OMCD28 and OMCTLA-4 have been described (26). cDNA inserts from these plasmids were subcloned into the  $\pi$ LN expression vector (provided by Dr. A. Aruffo, Bristol-Myers Squibb Pharmaceutical Research Institute) and transfected into COS cells (14). Cell surface expression was analyzed 48–72 h after transfection. CTLA4 (mur)Ig was a genetic fusion identical to CTLA4Ig (26), except that the human Ig portion was replaced with the analogous fragment from murine Ig C $\gamma$ 2a. Ig fusion proteins were purified from serum-free conditioned medium of transfected COS cells (14). Transfected COS cells were <sup>125</sup>I-surface labeled using lactoperoxidase, and H<sub>2</sub>O<sub>2</sub>, extracts were prepared and subjected to immunoprecipitation analysis as described (14). Cells cotransfected with OMCD28 and OMCTLA-4 were analyzed by two-color flow cytometry to determine the degree of coexpression of CD28 and CTLA-4. 34% of cells expressing CD28 also expressed CTLA-4, and 72% of cells expressing CTLA-4 expressed CD28.

**mAb Production.** BALB/c mice were primed subcutaneously with CTLA-4(mur)Ig (~50  $\mu$ g/mouse) in Titermax adjuvant (CytRx Corp., Norcross, GA). Secondary and tertiary immunizations were given intraperitoneally in PBS. 3 d after the third immunization, the spleen was removed and spleen cells were fused (34) with murine myeloma Ag8.653. Hybrids were screened on day 11 by enzyme immunoassays (EIA) for antibodies that bound immobilized CTLA4Ig. EIA reagents were obtained commercially (Genetic Systems Corp.). Cells in positive wells were cloned by limiting dilution.

**EIA.** For hybridoma screening, microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA) were coated with 1  $\mu$ g/ml CTLA4Ig for 16–24 h at 4°C. Wells were blocked for 1 h with sample diluent (Genetic Systems Corp.), then washed with PBS containing 0.05% Tween 20. Hybridoma supernatants were added and incubated at 23°C for 1 h. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig conjugate (Tago Inc., Burlingame, CA) was added and plates were again incubated for 1 h at 23°C. Finally, plates were washed thoroughly, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Genetic Systems Corp.) was added. For measurement of CTLA-4 and CD28 levels in cell extracts, microtiter plates were coated with mAb 11D4 or 9.3 at 1  $\mu$ g/ml in PBS. Nonionic detergent extracts of cells were prepared as for immunoprecipitation studies (14) and were added to plates for 1 h at 23°C. Plates were washed, and biotinylated mAb 7F8 or 9.3 were added for CTLA-4 or CD28 determinations, respectively. After an additional 1-h incubation, plates were washed again before addition of a HRP-streptavidin (SA) conjugate (Jackson Immunoresearch Laboratories, Inc., West Chester, PA). Plates were again incubated for 1 h, then washed extensively, and finally, developed by addition of o-Phenylenediamine (OPD) substrate (Sigma Chemical Co., St. Louis, MO). Enzyme reactions proceeded for ~30 min, and were stopped by the addition of an equal volume of 1 N H<sub>2</sub>SO<sub>4</sub>. Absorbance at 450 nm (TMB) or 490 nm (OPD) were recorded on a microtiter plate reader (Genetic Systems Corp.). The amount of CTLA-4 or CD28 in each sample was determined

<sup>1</sup> Abbreviations used in this paper: EIA, enzyme immunoassay; FBS, fetal bovine serum; HRP, horseradish peroxidase; LFE, linear fluorescence units; LSM, lymphocyte separation medium; PE-SA, R-phycoerythrin-conjugated streptavidin.

from a standard curve using known amounts of purified recombinant CD28Ig (14) or CTLA4Ig (26) as standards. The linear range of the CTLA-4 EIA was  $\sim 0.2$ – $10$  ng/ml CTLA4Ig, and the CD28 EIA,  $\sim 20$ – $1,000$  ng/ml CD28Ig. The CTLA-4 EIA showed no detectable crossreactivity with CD28Ig, and the CD28 EIA showed no detectable crossreactivity for CTLA-4. Neither assay gave significant signals with extracts from cells that did not express CTLA-4 (by RNA blot analysis) or CD28 (by cell surface staining).

**Immunostaining and Flow Cytometry.** Cells were collected by sedimentation and incubated with saturating amounts of mAbs in DMEM containing 10% FBS for 1–2 h at 4°C. When indirect immunofluorescence was measured, binding was detected by addition of a FITC goat anti-mouse Ig reagent (Tago Inc.), or by R-PE-conjugated SA (PE-SA, Molecular Probes, Inc., Eugene, OR). Cells were then analyzed on a flow cytometer (Epics C; Coulter Electronics Inc., Hialeah, FL).

**Cell Adhesion Assays.** T cell adhesion to B7<sup>+</sup> CHO cells was measured by modification of a published procedure (12). Monolayers of B7<sup>+</sup> CHO cells were fixed in situ with 0.5% paraformaldehyde in PBS for 20 min at 23°C. Cells were then washed and incubated in lymphocyte culture medium for 1 h before addition of T cells. Alloactivated CD4<sup>+</sup> T cells were <sup>51</sup>Cr-labeled and preincubated for 1 h at 23°C with or without mAbs in lymphocyte culture medium. Labeled T cells were added to B7<sup>+</sup> CHO monolayers, and adhesion reactions were initiated by brief centrifugation to bring cells in contact. Background cation-dependent adhesion was minimized by addition of EDTA (10 mM) to lymphocyte culture medium. Adhesion was allowed to proceed for 1–2 h at 37°C. Nonadherent cells were removed by washing monolayers four times with lymphocyte culture medium. Adhesion was quantified by measuring monolayer bound radioactivity in a gamma counter.

**RNA Blot Analysis.** RNA was prepared from stimulated PHA blasts ( $\sim 1$ – $3 \times 10^7$ /sample) by a rapid isolation procedure (35). RNA (10  $\mu$ g) was fractionated on formaldehyde agarose gels, transferred, and crosslinked to zeta probe (Bio-Rad Laboratories, Richmond, CA). Blots were prehybridized and hybridized with <sup>32</sup>P-labeled probes (36). CD28 probes were a 342-bp fragment corresponding to amino acids 4–117 of the predicted mature CD28 sequence; or full-length CD28 cDNA (37). The CTLA-4 probe was a 375-bp fragment corresponding to amino acids 1–125 of the predicted CTLA-4 protein sequence (22). The CD8 probe was a  $\sim 1.3$ -kb fragment of a CD8 cDNA clone provided by Dr. Jan Chalupny (Bristol-Myers Squibb Pharmaceutical Research Institute). The GAPDH probe has been previously described (14). DNA fragments were gel purified and <sup>32</sup>P-labeled using a random priming kit (Boehringer Mannheim Corp., Indianapolis, IN). Blots were sequentially hybridized with different <sup>32</sup>P-labeled probes. Between hybridizations, probe was stripped from the blots by boiling in a solution of SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS.

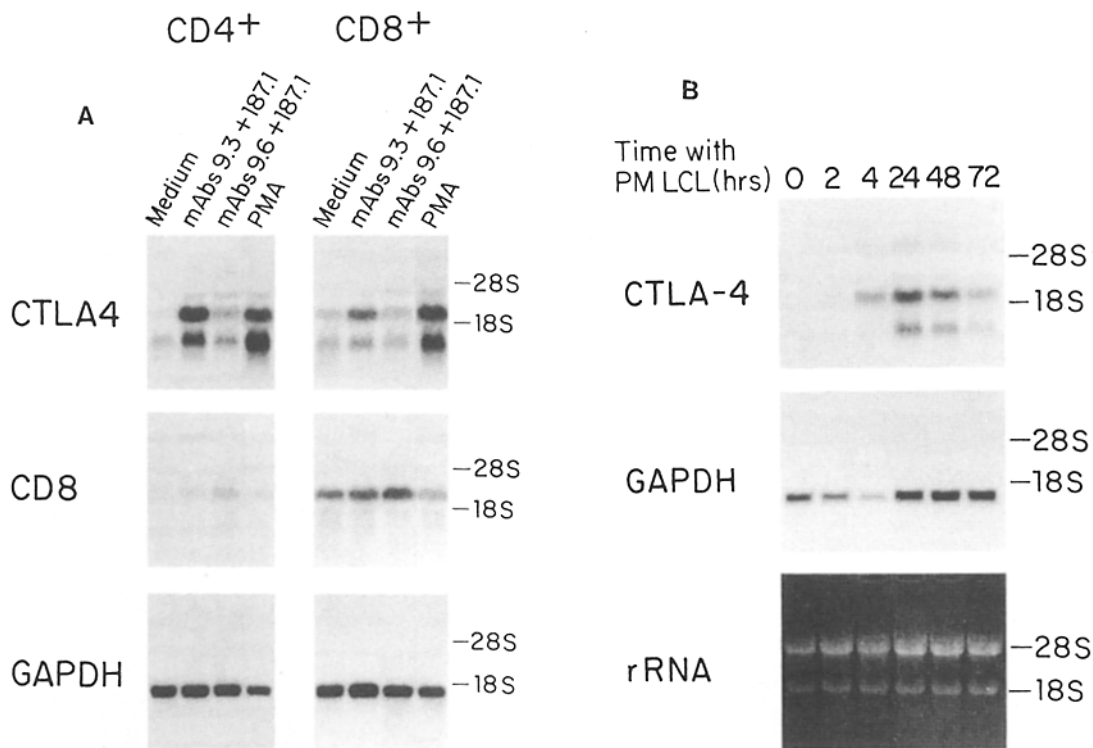
**MLC.** PBL were prepared by density gradient centrifugation on Ficoll-Hypaque. The cells were resuspended in medium containing RPMI 1640, 25 mM HEPES, 1 U/ml penicillin, 1  $\mu$ g/ml streptomycin, and 15% pooled human serum that had been heat inactivated at 56°C for 30 min. Responder and stimulator cells were unrelated individuals chosen so that there was at least one HLA class I and one HLA-DR antigen mismatched within each pair. Equal numbers ( $5 \times 10^4$  per well each) of responder and irradiated stimulator cells (3,000 rad) were cultured in the presence or absence of mAbs in round-bottomed 96-well plates. These were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. Assays were performed in triplicate. Cultures were pulsed with one  $\mu$ Ci of [<sup>3</sup>H]thymidine 18 h before harvesting.

## Results

**Regulation of Transcripts for CTLA-4.** We first compared the expression and regulation of CTLA-4 transcripts in CD4<sup>+</sup> or CD8<sup>+</sup> T cells. CD4<sup>+</sup> and CD8<sup>+</sup> enriched lymphocyte populations were obtained by complement-mediated lysis of CD8<sup>+</sup> and CD4<sup>+</sup> T cells, respectively, and the resulting populations were activated with PHA and rested as described in Materials and Methods. Transcripts for CTLA-4 were detected at similar levels in RNAs from both CD4<sup>+</sup> and CD8<sup>+</sup> subsets. Low levels of two CTLA-4 transcripts of  $\sim 1.8$  and  $\sim 0.6$  kb were detected in CD4<sup>+</sup> and CD8<sup>+</sup> enriched cells (Fig. 1 A). The  $\sim 1.8$ -kb transcript was reduced in the resting CD4<sup>+</sup> enriched population shown in Fig. 1 A, but not in another experiment. Both transcripts were increased in CD4<sup>+</sup> and CD8<sup>+</sup> enriched populations by stimulation with crosslinked anti-CD28 mAb and with PMA. Crosslinked anti-CD2 mAb was less effective at inducing CTLA-4 transcripts. Levels of CTLA-4 transcripts in alloantigen-primed CD4<sup>+</sup> cells at various times after activation were examined in Fig. 1 B. CTLA-4 transcripts were absent from rested alloantigen reactive T cells, but accumulated during activation, reached a peak at  $\sim 24$  h and thereafter decreased. Thus, CTLA-4 transcripts are expressed in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells after activation by CD28 engagement, by PMA, or by alloantigen activation.

**Preparation and Characterization of mAbs for CTLA-4.** To aid in studies of the distribution and function of CTLA-4, we prepared mAbs specific for this molecule as described in Materials and Methods. We selected a panel of 14 anti-CTLA-4 mAbs, of which three (11D4, 10A8, and 7F8) were selected for further study. All three mAbs were of the IgG1 isotype. In initial characterization experiments, these three mAbs bound strongly to CTLA-4-transfected COS cells or immobilized CTLA4Ig, but not to CD28-transfected COS cells or immobilized CD28Ig. Studies of mAbs binding to a panel of mutant CTLA4Ig proteins indicated that epitopes for these mAbs are dependent upon protein conformation rather than contiguous amino acid sequence (data not shown). These mAbs also inhibited binding of CTLA4Ig to B7<sup>+</sup> CHO cells (Fig. 2 A), with mAb 10A8 requiring lower concentrations to block equivalently (half-maximal inhibition at  $\sim 0.03$   $\mu$ g/ml, as compared with 0.1–0.2  $\mu$ g/ml for 11D4 and 7F8). This suggests that all three mAbs bind to biologically active (or “native”) CTLA-4 at or near the binding site for B7. In other experiments, mAb 10A8 competed for binding of biotinylated 11D4 to CTLA-4, but mAb 7F8 did not. This indicates that mAbs 11D4 and 10A8 bind spatially related epitopes, whereas the epitope for 7F8 was distinct.

mAb 11D4 was also characterized by immunoprecipitation analysis (Fig. 2 B). COS cells were transfected with OMCD28, OMCTLA-4, or a mixture of the two plasmids, <sup>125</sup>I-surface labeled and subjected to immunoprecipitation analysis with different mAbs and Ig fusion proteins. B7Ig and mAb 9.3, but not mAb 11D4, immunoprecipitated an  $\sim 72,000$ -*M<sub>r</sub>* protein (under nonreducing conditions) from CD28-transfected COS cells. This species is known from other experiments (14, 37) to be a disulfide-linked homodimer. Lesser



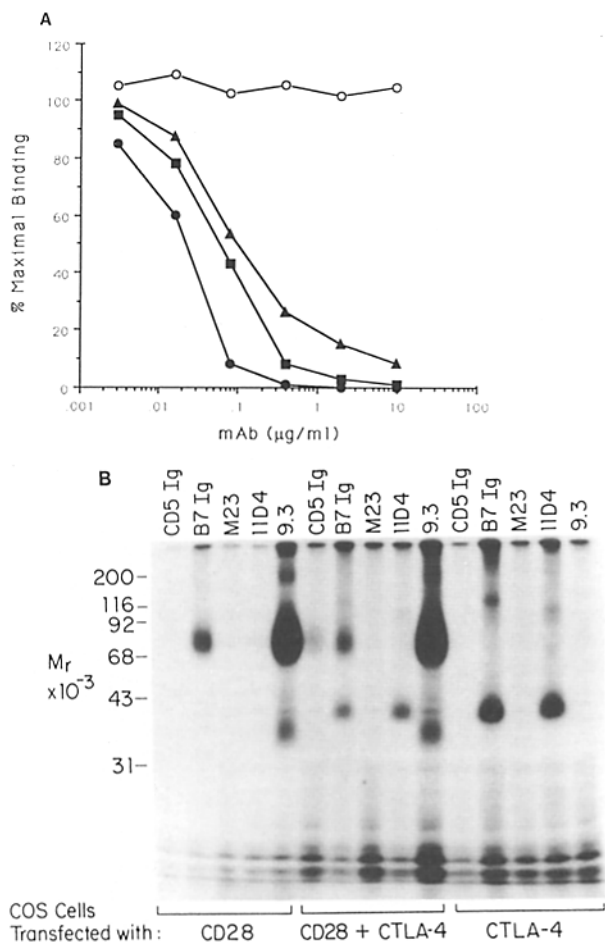
**Figure 1.** Regulation of CTLA-4 transcripts by T cell activation. (A) Regulation of CTLA-4 expression in different T cell subsets. PBL were depleted of CD8<sup>+</sup> or CD4<sup>+</sup> T cells and activated with PHA as described in Materials and Methods. Cells ( $2 \times 10^7$ ) were then stimulated for 4 h at 37°C with medium alone; with anti-CD28 mAb 9.3, or anti-CD2 mAb 9.6 at 2  $\mu\text{g}/\text{ml}$  for 10 min at 37°C followed by mAb 187.1 (antimurine Ig  $\kappa$  chain) at 10  $\mu\text{g}/\text{ml}$ ; or with PMA (10 ng/ml). Cells were harvested, and RNA was extracted and analyzed by blot analysis. The blot was sequentially hybridized with <sup>32</sup>P-labeled probes for CTLA-4, CD8, or GAPDH, as described in Materials and Methods. Migration positions are noted of the 28S and 18S ribosomal RNA species visualized by ethidium bromide staining. (B) Regulation of CTLA-4 transcripts during alloactivation. Resting alloantigen-primed CD4<sup>+</sup> T cells ( $2 \times 10^7$ ) were collected and restimulated with irradiated lymphoblastoid cells. Cells were harvested at the indicated times, and RNA was extracted and analyzed by blot analysis as described in Fig. 1 A. (Bottom) Ribosomal RNA bands visualized by ethidium bromide staining before transfer.

amounts of the nondisulfide-linked monomer (an  $\sim 37,000 M_r$ ) were also precipitated with the mAb. The  $\sim 72,000 M_r$  protein found in the lane precipitated with CD5Ig was due to spillover and was not seen in other experiments. mAb 9.3 was more effective at immunoprecipitation than B7Ig, which agrees with the greater affinity of the mAb (14). B7Ig and mAb 11D4 immunoprecipitated primarily an  $\sim 40,000 M_r$  protein from cells transfected with CTLA-4. In another experiment, we found proteins immunoprecipitated by B7Ig migrated with  $\sim 27,000 M_r$  upon reduction. B7Ig immunoprecipitated  $\sim 72,000$  and  $40,000 M_r$  proteins from COS cells transfected with both CD28 and CTLA-4. The larger of these was immunoprecipitated by mAb 9.3, and the smaller by mAb 11D4. Thus, mAb 11D4 specifically immunoprecipitates CTLA-4 from mixtures with CD28. These experiments provided no evidence of disulfide-linked complexes between CD28 and CTLA-4.

**Quantitation of CTLA-4 and CD28 Expression during Alloactivation.** Initial attempts to use anti-CTLA-4 mAbs to stain T cells from peripheral blood suggested that CTLA-4 was expressed at low levels. To monitor CTLA-4 expression, we used mAbs to nonoverlapping epitopes on CTLA-4 to develop a sandwich EIA for this molecule as described in

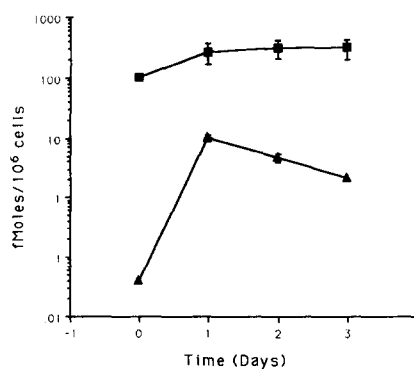
Materials and Methods. Biotinylated mAb 7F8 was used to detect CTLA-4 that had been immobilized by mAb 11D4. This assay could detect concentrations of CTLA4Ig of  $< 0.2$  ng/ml but gave no detectable signal at concentrations of CD28Ig of up to 10  $\mu\text{g}/\text{ml}$ . CTLA-4 concentrations were then determined in lymphocyte cell extracts by comparison with a standard curve constructed with known concentrations of CTLA4Ig. Extracts of activated lymphocytes that gave transcripts for CTLA-4 (PHA-activated T cell blasts, and activated CD4<sup>+</sup> cytotoxic T cell clones) tested positive in the CTLA-4 sandwich EIA, but cells that lacked CTLA-4 transcripts were negative (resting CD4<sup>+</sup> T cell clones, lymphoblastoid cell lines, T cell leukemias Jurkat and CEM). Expression of CTLA-4 protein during activation of alloantigen-primed CD4<sup>+</sup> cells (Fig. 3) followed the time course of transcript expression (Fig. 1 B). CTLA-4 was undetectable in resting cells ( $< \sim 0.04$  fmoles/ $10^6$  cells or  $\sim 25$  molecules/cell) and peak protein levels were measured at 1 d after activation ( $\sim 10$  fmoles/ $10^6$  cells or  $\sim 6,000$  molecules/cell). Thus, expression of CTLA-4 was induced  $\sim > 250$ -fold by alloactivation.

For comparison, CD28 levels in the same extracts were measured using a similar EIA (Fig. 3). In this case, mAb 9.3



**Figure 2.** Characterization of anti-CTLA-4 mAbs. (A) Anti-CTLA-4 mAbs block binding of CTLA4Ig to B7. Biotinylated-CTLA4Ig (10 ng/ml final concentration) was incubated for 1 h at 23°C with the indicated concentrations of anti-CTLA-4 mAbs 7F8 (■), 10A8 (●), or 11D4 (▲); or with isotype control mAb M23 (○). B7<sup>+</sup> CHO cells were removed from their culture vessel by incubation in PBS containing 10 mM EDTA, collected by centrifugation, incubated with the mAb/biotinyl-CTLA4Ig mixture for ~1 h at 23°C, and washed. Binding of biotinyl-CTLA4Ig was then detected with PE-SA, and cells were analyzed by flow cytometry. Mean fluorescence values were recorded and converted to linear fluorescence units (LFU). Background fluorescence (~2 LFU) was subtracted from all values. Values are expressed as percentage of maximal fluorescence in the absence of mAb (~13 LFU). (B) Immunoprecipitation of CTLA-4 from transfected COS cells. COS cells (~10<sup>6</sup>) were transfected with equal amounts of expression plasmids OMCD28, OMCTLA-4, or a mixture of these two plasmids. 48 h after transfection, cells were detached from their culture dishes with EDTA, and subjected to cell surface iodination and immunoprecipitation analysis (14) with the indicated Ig molecules at 20 µg/ml. CD5Ig (human IgG1) and mAb M23 (murine IgG1) were used as controls. SDS-PAGE was performed on a 10–20% acrylamide gel run under nonreducing conditions. The migration of standard proteins is indicated.

was used as both capture and detecting mAb, and CD28Ig was used as standard. The ability of mAb 9.3 to both capture and detect CD28 probably reflects that this molecule is homodimeric. The CD28 sandwich EIA did not show reactivity with CTLA-4Ig at concentrations of up to 10 µg/ml, nor did it show reactivity with extracts from a lymphoblastoid



**Figure 3.** Ratio of CTLA-4 to CD28 increases during alloactivation of primed CD4<sup>+</sup> cells. Resting, viable-primed CD4<sup>+</sup> cells were restimulated with irradiated lymphoblastoid cells. At the indicated times, cells were collected, extracted with nonionic detergent (14), and amounts of CD28 (■) and CTLA-4 (▲) in the extract were measured by sandwich EIAs, as described in Materials and Methods. Shown are means and SD (bars) of measurements made with three serial dilutions that fell within the linear region of standard curves for each sample. The value for CTLA-4 at day 0 represents a maximal estimate since this measurement was below the linear portion of the standard curve.

cell line that does not express CD28. CD28 levels in extracts from antigen-primed CD4<sup>+</sup> T cells also increased during activation, but less than those for CTLA-4. The ratio of CTLA-4 to CD28 therefore increased during alloactivation, reaching a maximum of ~1:30 between 1 and 2 d after activation.

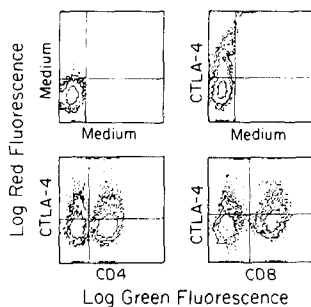
**Cell Surface Expression of CTLA-4 on Activated T Cells.** Having determined the kinetics of CTLA-4 expression, we next investigated its surface expression. Preliminary experiments showed that when alloactivated, primed CD4<sup>+</sup> T cells were stained with biotinylated mAb 11D4 and analyzed by flow cytometry, the intensity of staining with mAb 11D4 varied depending upon light scatter profile of the population analyzed. Staining was brighter on a subpopulation of larger activated cells. This staining was specific since unlabeled mAb 11D4 (but not an isotype control mAb) competed for staining by biotinylated mAb 11D4. The main population of activated cells and resting cells stained less intensely. Thus, surface expression of CTLA-4 was greater on larger, activated cells. Similar findings are demonstrated below in Fig. 5.

Surface expression of CTLA-4 was also detected on PBL activated with immobilized anti-CD3 mAb, and on purified CD4<sup>+</sup> T cells activated with immobilized anti-CD3 mAb or with a coimmobilized combination of anti-TCR mAb and ICAM-1 Rg (33). Surface expression of CTLA-4 was not detected on resting cells, but became detectable after 2–4 d of activation. In all cases, staining was brighter on a population of larger, activated cells which comprised 5–20% of the total cell population, of which up to ~50% stained with mAb 11D4. When PBLs activated for 3 d with immobilized anti-CD3 were pulse labeled with BrdU and stained with FITC-labeled anti-BrdU mAb (38), the larger activated population had an ~two-fold higher percentage of stained cells (in S phase) than for the bulk population. No difference in BrdU incorporation was seen in 11D4 positive or negative

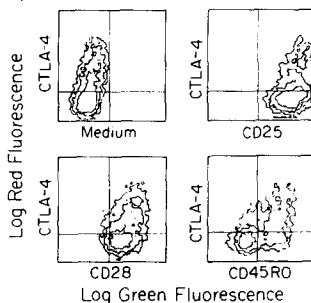
cells, indicating that CTLA-4 expression is not restricted to cells in S phase. The distribution of CTLA-4 on different T cell subsets was examined in the experiment shown in Fig. 4. Results presented are for the larger activated subpopulation, although qualitatively similar results were also seen when the total population was analyzed. CTLA-4 surface expression was detected on CD4<sup>+</sup> and CD8<sup>+</sup> cells. CTLA-4 was coexpressed with CD25, CD28, and CD45RO. Thus, CTLA-4 is expressed on a fraction of the activated CD28<sup>+</sup> T cells of both CD4<sup>+</sup> and CD8<sup>+</sup> subsets.

**Cooperation of CTLA-4 and CD28 in Mediating Adhesion to B7.** Since CTLA-4 and CD28 were coexpressed on T cells, it was important to determine the relative contributions of these molecules towards adhesion to B7. In previous studies (26), we showed that CTLA4Ig bound B7 with greater avidity than did CD28Ig, which suggested that CTLA-4 is a higher avidity receptor for B7 than is CD28. Expression of CTLA-4 on activated T lymphocytes might then be expected to increase their binding to B7. We took two approaches towards testing this possibility. In one approach, CD4<sup>+</sup> T cells from PBL were activated by coimmobilized anti-TCR plus ICAM-1 Rg and tested for their ability to bind biotinylated B7Ig (Fig. 5). Large, activated cells expressed more CTLA-4 and also expressed approximately twofold more CD28 than the smaller cell population. Comparison of mean fluorescence values indicated that large, activated cells expressed ~50-fold more CD28 than CTLA-4, assuming equivalent detection of the two mAbs. Large, activated cells also bound more B7Ig (Fig. 5). Binding of B7Ig to both large and small cell populations was stronger when the concentration of biotinylated B7Ig was increased, but for this experiment the concentration of B7Ig used was minimized so as to more easily compete for B7Ig binding with mAbs. B7Ig binding was blocked by both

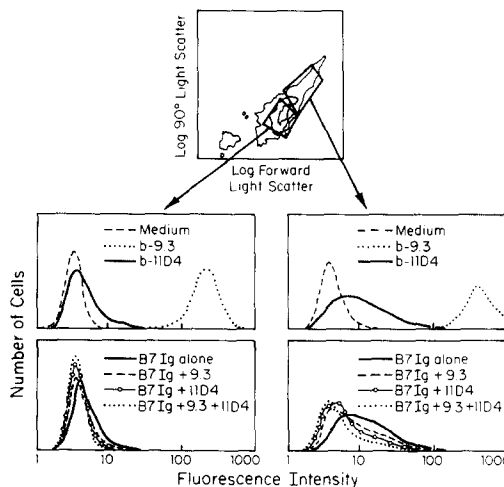
#### Experiment 1



#### Experiment 2



**Figure 4.** CTLA-4 expression by subsets of anti-CD3 activated T lymphocytes. PBL were activated by incubation with immobilized anti-CD3 mAb for 2 d at 37°C. Cells were collected and stained without or with biotinylated mAb 11D4. Cells were washed and then incubated with PE-SA without or with FITC-conjugated mAbs G17.2 (anti-CD4), 10.1 (anti-CD8), 7G7/B6 (anti-CD25), 9.3 (anti-CD28), or UCHL-1 (anti-CD45RO). Cells were then subjected to two-color analysis by flow cytometry. Shown are contour plots obtained from two independent experiments. Results shown are from larger activated cells, although qualitatively similar results were also obtained with smaller cells. Staining with biotinylated mAb 11D4 was inhibited by prior incubation of cells with excess unlabeled mAb 11D4, but not with an excess of isotype-matched control mAb.

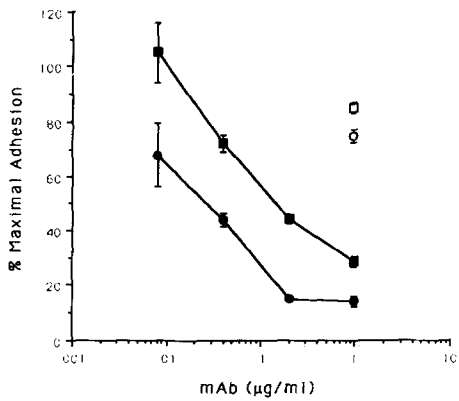


**Figure 5.** B7 binds better to T lymphocytes expressing the CTLA-4 counter receptor. CD4<sup>+</sup> T cells were activated by culturing for 3 d with coimmobilized anti-TCR mAb WT31 with ICAM-1 Rg fusion protein. Cells were collected and stained with medium alone, or with biotinylated mAbs 9.3 or 11D4 (middle) or with biotinylated B7Ig (bottom), all at 10 µg/ml. Where indicated, binding of biotinylated B7Ig was blocked by preincubation with an excess (100 µg/ml) of unlabeled mAbs 9.3, 11D4, or with 9.3 plus 11D4. B7Ig binding was detected by addition of PE-SA, and analyzed by flow cytometry. Control experiments showed that binding of biotinylated-B7Ig was blocked by an excess of unlabeled B7Ig, but not by an irrelevant human Ig, or by an irrelevant murine IgG1 mAb.

mAbs 11D4 and 9.3, but not by a control mAb of irrelevant specificity. Identical results were obtained when PBL were activated with immobilized anti-CD3 mAb before staining. These findings indicate that large activated cells bind more B7 than smaller cells, and that both CTLA-4 and CD28 contribute to this increased binding, despite the great difference in abundance of the two molecules.

We also examined the relative contributions of CTLA-4 and CD28 on activated T cells towards binding B7 using a cellular adhesion assay. In these experiments, antigen-primed CD4<sup>+</sup> T cells were alloactivated, <sup>51</sup>Cr-labeled, and tested for their ability to adhere to B7<sup>+</sup> CHO cells in the presence of increasing amounts of anti-CD28 mAb 9.3 or a combination of anti-CD28 mAb 9.3 plus anti-CTLA-4 mAb 11D4 (Fig. 6). mAb 9.3 inhibited CD4<sup>+</sup> T cell adhesion to B7<sup>+</sup> CHO cells in a dose-dependent fashion. mAb 11D4 alone at 1 µg/ml did not significantly affect adhesion when compared with an irrelevant control mAb. The addition of 1 µg/ml mAb 11D4 to increasing concentrations of mAb 9.3 resulted in a shifting of the dose-response curve approximately five-fold to the left. Thus, mAb 11D4 was synergistic with mAb 9.3 at inhibiting adhesion of activated T cells to B7. Identical results were obtained in three different experiments. These data indicate that CTLA-4 and CD28 cooperatively mediate adhesion to B7.

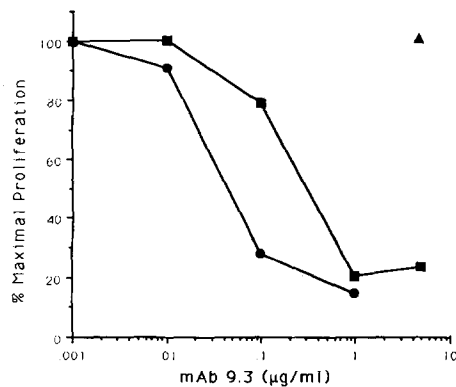
**Functional Cooperation between CTLA-4 and CD28 during T Cell Activation.** The involvement of CTLA-4 and CD28 in costimulation during primary MLC was examined (Fig. 7). Anti-CD28 and anti-CTLA-4 mAbs were used alone and in combination to block involvement of these molecules in



**Figure 6.** Anti-CD28 and anti-CTLA-4 mAbs cooperatively inhibit adhesion of activated CD4<sup>+</sup> cells to B7<sup>+</sup> CHO cells. Adhesion of alloactivated (24 h) <sup>51</sup>Cr-labeled CD4<sup>+</sup> T cells to fixed monolayers of B7<sup>+</sup> CHO cells was measured as described in Materials and Methods. T cells were incubated with 1 μg/ml anti-CTLA-4 mAb 11D4 (○) or isotype control mAb M23 (□); or with the indicated concentrations of mAb 9.3 mixed with 1 μg/ml mAbs 11D4 (●) or M23 (■) before initiation of adhesion. Adhesion was allowed to proceed for 1 h at 37°C, and nonadherent cells were removed by washing. Monolayers were solubilized by addition of 1 N NaOH, and bound radioactivity was measured with a gamma counter. Values are expressed as a percentage of adhesion measured in the absence of mAb (5,400 cpm, 3,800 cells, or ~6% of input cells).

proliferation during primary MLC. In these experiments, Fab fragments of mAb 9.3 were used since these are more efficient inhibitors of MLC (39). mAb 11D4 at 5 μg/ml did not inhibit primary MLC, but increased by ~10-fold the ability of various concentrations of Fab fragment of mAb 9.3 to inhibit MLC. These data suggest that CTLA-4 and CD28 cooperatively regulate T cell proliferative responses during MLC.

Experiments were performed to demonstrate effects of anti-CTLA-4 mAbs on T cell proliferation. We observed that,



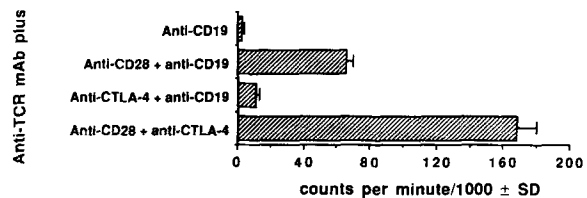
**Figure 7.** Anti-CD28 and anti-CTLA-4 mAbs cooperatively inhibit primary MLC. Primary MLC with HLA-DR mismatched responder and stimulator cells was performed in the presence of 5 μg/ml of anti-CTLA-4 mAb 11D4 alone (▲); or with the indicated concentrations of Fab fragment of anti-CD28 mAb 9.3 alone (■) or together with 5 μg/ml of mAb 11D4 (●). Means of cpm incorporation of three replicates were calculated. Data for each treatment are represented as a percentage of the peak proliferative response in untreated cultures (30,500 cpm).

in contrast to anti-CD28 mAb 9.3 (6), mAb 11D4 did not significantly augment anti-CD3 or PMA-induced proliferation of PBL during 3-d cultures. Since CTLA-4 expression is activation dependent, we also tested the ability of mAb 11D4 to augment proliferation of T cells that had been preactivated for 3 d with anti-TCR mAb and ICAM-1 Rg (33). Coimmobilization of anti-TCR mAb and anti-CD28 mAb 9.3 greatly increased proliferation of these cells (Fig. 8). Coimmobilized combinations of anti-TCR mAb and anti-CTLA-4 mAb 11D4 increased proliferation slightly. In some experiments, this combination of mAbs gave greater enhancement of proliferation, but always less than the combination of anti-TCR mAb plus anti-CD28 mAb. Coimmobilization of anti-TCR mAb plus a combination of mAbs 9.3 and 11D4 gave synergistic effects on proliferation. When anti-TCR mAb was omitted, proliferation was insignificant (data not shown), indicating that the proliferative effects of mAbs 11D4 and 9.3 also required TCR activation. Similar results were obtained with anti-CTLA-4 mAb 10A8. Thus, mAbs to CD28 and CTLA-4 were synergistic in their ability to costimulate preactivated CD4<sup>+</sup> T cells.

## Discussion

CTLA-4 was identified as a transcript encoding a "cytotoxic T lymphocyte-associated" molecule (20). Here we have shown that CTLA-4 is expressed at similar levels on both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets, and is coexpressed with the CD25 activation marker. CTLA-4 expression is regulated in similar fashion on both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. Expression of CTLA-4 is therefore not restricted to cytotoxic T cells, but rather more generally, to activated T lymphocytes. Our results also show that CTLA-4 and CD28 are not generally expressed on reciprocal subsets of T cells, as has been speculated (40). Instead, CTLA-4 was coexpressed with its homologue CD28 on a subset of activated T cells.

Cells that expressed the highest levels of CTLA-4 (detected by mAb 11D4) also bound more of the B7Ig fusion protein, partly as a result of CTLA-4 expression, since B7Ig binding



**Figure 8.** Anti-CTLA-4 and anti-CD28 mAbs synergistically costimulate proliferation of primed CD4<sup>+</sup> T lymphocytes. CD4<sup>+</sup> T cells from PBL were activated for 3 d with coimmobilized anti-TCR mAb WT31 with ICAM-1 Rg fusion protein as described in Fig. 5. Cells were then collected and cultured with 20 ng/well WT31 (anti-TCR) plus 40 ng/well negative control mAb 4G7 (anti-CD19); or 20 ng/well each of mAb 4G7 and mAbs 9.3 (anti-CD28) or 11D4 (anti-CTLA-4); or with 20 ng/well each of mAbs 9.3 and 11D4. Proliferation was measured by addition of [<sup>3</sup>H]thymidine (1 μCi/well) during the final 16 h of a 3-d culture at 37°C. Shown are results from triplicate cultures (± SD). Wells from which anti-TCR mAb was omitted gave <1,000 cpm.

was partly blocked by mAb 11D4. Thus, although CTLA-4 was expressed much less than CD28 on this cell population ( $\sim 50$ -fold less) it bound a significant fraction of the total B7Ig. This is consistent with CTLA-4 having higher avidity for B7 (26).

CTLA-4 is expressed much less abundantly than its homologue, CD28. Expression was induced several hundred-fold upon activation. This finding is consistent with earlier studies (20, 23) which demonstrated an increase in CTLA-4 transcript levels upon T cell activation. Even at a maximum, CTLA-4 was expressed at only  $\sim 1/30$ –50 the levels of CD28, as estimated in measurements of total (intracellular plus cell surface) CTLA-4 (Fig. 3), and cell surface CTLA-4 (Fig. 5). The difference in expression between CD28 and CTLA-4 supports the earlier conclusion (14) that CD28 is the primary counter receptor for B7 on PHA-activated T cell blasts.

Taken together, these results indicate that CTLA-4 is an activation-regulated counter receptor of high avidity and low abundance. CD28, on the other hand, is expressed on both resting and activated cells, and has lower avidity for B7, but higher abundance. The overall degree of interaction between B7 costimulatory molecules on APC, and CD28 and CTLA-4 on activated T cells would be determined by the relative levels of the two T cell molecules. It is intriguing that the difference in expression levels between CD28 and CTLA-4 on activated cells (30–50-fold) is similar in magnitude to the difference in avidities of their respective Ig fusion proteins for B7 ( $\sim 20$ -fold; reference 26). This suggests that the difference in avidities for B7 between these molecules is delicately balanced on activated T cells by differences in their levels of expression.

Combinations of mAbs to CD28 and CTLA-4 cooperatively inhibit adhesion of activated T cells to B7. Blocking CTLA-4 interactions with B7 therefore increased the ability of anti-CD28 mAbs to block CD28 interactions with B7. This suggests that CD28 and CTLA-4 cooperatively regulate adhesion to B7. Anti-CTLA-4 mAbs alone were poor inhibitors of T cell adhesion or proliferation in MLC even though these mAbs blocked binding of CTLA4Ig to B7. Anti-CTLA-4 mAbs were also relatively inefficient at promoting T cell activation when coimmobilized with anti-TCR mAb. CTLA-4 interactions with B7 therefore are neither necessary nor sufficient for T cell adhesion or activation by B7. Conversely, CD28 mAbs are good inhibitors of adhesion and

proliferation in MLC (39), and are potent costimulators of T cell proliferation (6). This suggests that CD28–B7 interactions are essential for T cell activation by APC.

Our data suggest that one function of CTLA-4 on activated T cells may therefore be to promote effective interactions between CD28 and B7. Low abundance, high avidity CTLA-4 molecules appear to facilitate interactions and/or signals between higher abundance, lower avidity CD28 molecules and the B7 counter receptor. One model to explain these data would be in which CTLA-4 and CD28 exist on the T cell surface in microdomains, as has been demonstrated for other adhesion molecules (41). The inclusion of CTLA-4 in such microdomains might increase the probability of interaction between CD28 and B7 on the APC. Alternatively, B7 might exist in microdomains, as has been suggested by immunofluorescence studies of certain APC (Symington, F., W. Brady, and P. S. Linsley, unpublished observations). CTLA-4 might then bind to such a microdomain and thereby increase the probability that it would interact with CD28 on the T cell surface. A more complete understanding of the molecular mechanisms by which CTLA-4 facilitates T cell activation by CD28 will require careful investigation of the topography of CD28 and CTLA-4 on T cells, and of B7 on APC.

Anti-CTLA-4 mAbs were less effective costimulators of T cell proliferation than anti-CD28 mAb 9.3. We have also been unable to trigger intracellular calcium mobilization or increases in IL-2 mRNA with anti-CTLA-4 mAbs under conditions that lead to clear signals with the anti-CD28 mAb 9.3 (Greene, J., N. R. Demle, and P. S. Linsley, unpublished observations). This may result in part from the lower expression of CTLA-4, but it may also indicate that signals transmitted by these two receptors are qualitatively different. The cytoplasmic domains of CD28 and CTLA-4 show somewhat limited sequence identity (26–29% for murine and human molecules; 23), possibly suggesting different interactions with signal-transducing molecules. It may also be significant that the cytoplasmic domains of murine and human CTLA-4 are identical (22, 23), whereas those from murine and human CD28 are only  $\sim 77\%$  identical (40). This may reflect evolutionary pressure to conserve an essential signal-transducing function for CTLA-4. The anti-CTLA-4 mAbs we have described will facilitate future investigations on signal(s) transduced by this molecule.

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