Pillars Article: CTLA-4 Can Function as a Negative Regulator of T Cell Activation.


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CTL4 Can Function as a Negative Regulator of T Cell Activation

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Summary

CD28 and CTLA-4 are related glycoproteins found on T cells. Ligation of CD28 following antigen receptor engagement provides a costimulatory signal required for T cell activation. Anti-CTLA-4 antibodies were generated to examine the role of the CTLA-4 receptor on murine T cells. Expression of CTLA-4 as a homodimer is up-regulated 2-3 days following T cell activation. Anti-CTLA-4 antibodies and Fab fragments augmented T cell proliferation in an allogeneic MLR. However, when optimal costimulation and Fc cross-linking were present, anti-CTLA-4 mAbs inhibited T cell proliferation. Together, these results suggest that the mAb may obstruct the interaction of CTLA-4 with its natural ligand and block a negative signal, or directly signal T cells to down-regulate immune function.

Introduction

The activation of T lymphocytes and subsequent generation of effector activity are dependent on at least two signals delivered by antigen-presenting cells (APC). The first signal is delivered when the T cell receptor (TCR) is ligated by the antigenic peptide-major histocompatibility complex (MHC) molecular complex. There is also a requirement for costimulatory signals mediated by the interaction of TCR-independent T cell surface molecules and ligands expressed on the APC. The interaction of CD28 on T cells with either B7-1 (CD80) or B7-2 (CD86) on APC has been shown to provide one such costimulatory signal for helper T cells to produce interleukin-2 (IL-2) (June et al., 1990, 1994; Freeman et al., 1993a; Lenschow et al., 1993).

CTLA-4 is a molecule that was cloned from a subtractive library derived from a CTL clone (Brunet et al., 1987). The CTLA-4 gene colocalized to the same chromosome as CD28 in both mouse and human (Lafage-Pochitaloff et al., 1990; Harper et al., 1991). The predicted amino acid sequence of the gene was homologous to CD28, especially in the transmembrane domain (Harper et al., 1991). Most importantly, several structural motifs are shared between the two amino acid sequences, including a sequence, MYPPP, which is predicted to allow binding of the CD28 molecule to the B7-1 molecule (Harper et al., 1991) and to B7-2 by a number of investigators (Lenschow et al., 1993; Freeman et al., 1993a, 1993b; Azuma et al., 1993). The binding of CTLA-4 to B7 was demonstrated by Linsley and colleagues (1991) using a soluble form of the molecule, made up of the extracellular domain of CTLA-4 and the Fc portion of a human immunoglobulin G1 (IgG1), CTLA-4lg. In fact, binding studies have demonstrated that CTLA-4lg has a 10- to 20-fold higher affinity for B7-1 than a similarly derived fusion protein with the extracellular domain of CD28 (Linsley et al., 1991). CTLA-4lg is also capable of blocking in vitro proliferation in assays of alloreactivity (Linsley et al., 1991; Lenschow et al., 1993), as well as preventing xenograft rejection (Lenschow et al., 1992) and prolonging allograft rejection (Turka et al., 1992; Lin et al., 1993), presumably by preventing reactive T cells from receiving their required second signal. In addition, CTLA-4lg also has inhibitory effects on immunoglobulin production (Linsley et al., 1992b; Lane et al., 1994).

However, in spite of the similarities between the extracellular binding domains of the CD28 and CTLA-4 glycoproteins, a number of differences suggest these molecules may participate in distinct ways during an immune response. First, CTLA-4 mRNA transcripts are virtually undetectable in resting human and murine T cells, and increase only after T cell activation. In activated human T cells, transcripts can be detected as early as 1 hr following stimulation by TCR- and CD28-mediated signals (Lindsten et al., 1993). CTLA-4 mRNA is also detected in activated murine T cells and T cell clones (Freeman et al., 1992). In fact, recent studies by Linsley et al. (1992a) have documented cell surface expression of CTLA-4 on activated human cells using a mAb specific for human CTLA-4. Expression of CTLA-4 on the surface of peripheral blood lymphocytes (PBLS) was restricted to the T cell blasts, 2-4 days following activation.

Although current research has increased our understanding of the regulation of CTLA-4 mRNA and surface expression, the functional role of CTLA-4 remains unclear. Initial studies have suggested that CTLA-4 may function as an additional costimulatory molecule. Linsley et al. (1992a) demonstrated that anti-human CTLA-4 MAbs increased proliferation of T cells stimulated with anti-CD3 plus anti-CD28. However, these studies did not rule out other functional roles of CTLA-4 during natural immune responses. The present study was initiated to develop reagents specific for murine CTLA-4 to address further the role of this molecule in the regulation of T cell activation.
Results

CTLA-4 Is Detected Only on Activated Murine T Lymphocytes Following Stimulation with Anti-CD3 or Antigen

To examine CTLA-4 protein expression on murine T lymphocytes, hamster MAbs were generated with a murine CTLA-4 Ig fusion protein. Candidate hybridomas were screened by using the CTLA-4 Ig molecule as a capture substrate for hybridoma supernatants in an enzyme-linked immunosorbent assay. The specificity of three MAbs, UC10-1B8, UC10-4B8-12, and UC10-4F10-11 (4F10) were confirmed on CHO cells transfected with a glycosylphosphatidylinositol (GPI)-linked form of the CTLA-4 extracellular domain (data not shown). All of the antibodies stained CTLA-4GPI-CHO transfectants, but not untransfected CHO cells. The 4F10 MAb routinely stained at the highest levels and was utilized for the majority of subsequent experiments.

Previous experiments have demonstrated that CTLA-4 mRNA transcripts are induced following activation of human or murine T lymphocytes. However, in the murine model, direct evidence of CTLA-4 protein expression has not been established. To examine murine T cells for CTLA-4 expression, splenocytes from B6 mice were incubated with soluble anti-CD3 at 10 μg/ml and examined for CTLA-4 expression on both the CD4+ and CD8+ T cell subsets at 48 hr (Figure 1, top left and top right, respectively). Resting cells, either CD4+ or CD8+, showed no evidence of cell surface CTLA-4 expression (data not shown). However, 48 hr following activation with anti-CD3, both CD4+ and CD8+ cells expressed CTLA-4. CD8+ T cells expressed higher levels of CTLA-4 than CD4+ cells. In both subsets, the large blast cells expressed the highest level of CTLA-4 (data not shown).

Previously, it has been shown that CTLA-4 mRNA transcripts are increased when human PBL are stimulated with both anti-CD3 and anti-CD28 as opposed to anti-CD3 alone (Lindsten et al., 1993). However, it has also been suggested, using human PBL, that CD28 ligation is not critical for CTLA-4 expression (Damilakis et al., 1994). To look at the dependence of CTLA-4 expression on CD28 signaling, splenocytes from CD28 (−/−) and B6 (wild-type) mice were activated either with anti-CD3 alone at 10 μg/ml or anti-CD3 in the presence of 50 U/ml IL-2 (Figure 1, bottom left and bottom right). CD4+ and CD8+ T cells from the CD28 (−/−) mice expressed minimal levels of CTLA-4 in the presence of soluble anti-CD3 alone as compared with wild-type mice. However, CD28 (−/−) splenic T cells cultured in the presence of anti-CD3 plus IL-2 expressed significant levels of CTLA-4, albeit lower levels than observed on wild-type splenic T cells under either stimulation condition (Figure 1). These results suggest that CD28 expression is required for optimal CTLA-4 expression on the surface of T cells. The essential signals delivered through the CD28 receptor could be partially replaced by IL-2, implying that at least one important element of CD28-dependent CTLA-4 expression is the regulation of IL-2 through the CD28 pathway.

Kinetics of CTLA-4 Expression on Anti-CD3 and Antigen-Activated Murine T Lymphocytes

The functional role of CTLA-4 in T cell responses is likely to be a function of its time of expression. Therefore, the kinetics of CTLA-4 expression following exposure to soluble anti-CD3 MAb or nominal antigen stimulation was examined (Figure 2). CTLA-4 expression is first detected at 24 hr following stimulation of T cells with anti-CD3 MAb. Peak expression was observed at 48 hr. By 96 hr, little, if any, CTLA-4 was detected on the surface of activated cells (Figure 2A). To determine whether stimulation of T cells with a nominal antigen resulted in a similar time course of CTLA-4 expression, H-2Ld-reactive T cells from the 2C TCR transgenic mice were stimulated with the H-2Ld-bearing P815 mastocytoma cells transfected with murine B7-1 (P915/B7-1). Up-regulation of CTLA-4 on clonotype-positive T cells, stimulated with the H-2Ld alloraggen, followed a time course similar to that previously observed with anti-CD3 (Figure 2B). Maximal expression of CTLA-4 was observed at 48 hr following activation. These
findings demonstrated that CTLA-4 is expressed on antigen-reactive T cells following activation by MHC-restricted antigenic signal in the presence of a costimulatory signal.

**CTLA-4 Exists Predominantly as a Homodimer on the Surface of Activated T Cells**

To examine the biochemical nature of the CTLA-4 glycoprotein expressed on activated T cells, splenocytes from B6 mice were stimulated using anti-CD3 and IL-2, cultured for 72 hr, harvested, passed over Ficoll to remove dead cells, and cell surface labeled with biotin. Labeled cell lysates were immunoprecipitated with either a negative control hamster antibody (UC3-10A6) or the anti-CTLA-4 MAb, 4F10, and analyzed by two-dimensional nonreduced/reduced polyacrylamide gel electrophoresis as described in the Experimental Procedures. Figure 3 illustrates that the CTLA-4 glycoprotein is expressed on activated murine T cells as a dimer composed of one of two molecules, either 33-34 kDa or 36-37 kDa. Treatment of the anti-CTLA-4 immunoprecipitate with N-glycanase resulted in a size reduction of both glycoprotein species to the same core size of approximately 21 kDa (data not shown). These results suggest that CTLA-4 can exist as a homodimer of differentially glycosylated forms. A nondisulfide-linked form of CTLA-4 was observed migrating on the diagonal at the same glycosylated and core molecular mass as the smaller of the two species of CTLA-4 dimer. This nondisulfide-linked dimeric or monomeric form was observed on activated normal and CD28-deficient T cells. It has been hypothesized that CTLA-4 may exist on the cell surface dimerized with CD28, given the amino acid...
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Figure 4. Anti-CTLA-4 MAb Can Augment Proliferation in an Allogeneic MLR.

(A) Purified 2C T cells (2 x 10^5 cells/well) were cultured with the indicated numbers of allogeneic γ-irradiated DBA/2 splenocytes. Cells were cultured either with 4F10 MAb at 100 μg/ml final concentration (open bars) or without added MAb (closed bars). Cells were harvested 96 h following the initiation of culture. Data is representative of three experiments.

(B) Purified 2C TCR transgenic T cells (2 x 10^5/well) were cultured in the presence of 5 x 10^5/well γ-irradiated (2000 rad) BALB/c splenocytes. All reagents were added at a 50 μg/ml final concentration. In no case was augmentation seen in the presence of syngeneic (B6) feeder cells. This figure is representative of data from four experiments.

(C) Purified 2C T cells (2 x 10^5 cells/well) were cultured with 1 x 10^6 allogeneic γ-irradiated DBA/2 splenocytes in the presence of either negative control hamster MAb, GL4 (100 μg/ml) or the indicated concentration of 4F10. Assays were harvested at 96 h following initiation of culture. Data is representative of five experiments.

Figure 5. 4F10 Can Augment Proliferation in the Presence of Increased TCR Stimulation.

Allogeneic irradiated BALB/c stimulators (2.5 x 10^7 cells/well) were cultured with the octapeptide (LSPFFRDL) at the indicated concentrations for 1 hr at 37°C. 4F10 or a control hamster immunoglobulin were added at a final concentration of 50 μg/ml and 2 x 10^6 peptide-specific purified 2C TCR transgenic T cells were added. Assays were harvested 96 h after the initiation of culture. Data are representative of three experiments.

Antibodies Against CTLA-4 Can Augment an Allogeneic Response

The anti-CTLA-4 antibodies were examined functionally in an allogeneic in vitro T cell proliferative responses. T cells were purified from the lymph nodes of 2C TCR transgenic mice and stimulated with graded doses of irradiated allogeneic DBA/2 splenocytes. The anti-CTLA-4 MAb, 4F10, had a significant augmenting effect on T cell proliferation over a wide range of stimulation conditions (2- to 10-fold; Figure 4A). However, the greatest fold augmentation of proliferation was observed at the lower concentrations of stimulators. Therefore, further experiments were performed using reduced stimulators to examine the augmented proliferative response. As seen in Figure 4B, several different anti-CTLA-4 MAba augmented the proliferative responses between 2- to 5-fold above responses observed in cultures treated with control hamster immunoglobulin (Figure 4B) or other hamster MAb (data not shown). The most dramatic augmentation was observed following the addition of the 4F10 MAb, perhaps reflecting its epitope specificity, immunoglobulin subclass or relative affinity as compared with the other anti-CTLA-4 MAb. The ability of anti-CTLA-4 MAb to augment T cell proliferation depended on an initial signal, presumably delivered via the TCR, inasmuch as the MAb had no effect on the proliferative response of purified responder T cells to syngeneic B6 APCs (data not shown). Similar levels of enhanced proliferation were observed in allogeneic mixed lymphocyte reaction (MLR) assays using purified B6 T cells as the responder population (data not shown). In Figure 4C, the 4F10 MAb was titrated and examined for its ability to augment proliferation over a range of MAb concentration. Significant augmentation of the T cell proliferative response was observed upon the addition of as little as 8.25 μg/ml. Thus, anti-CTLA-4 MAb were able to augment T cell proliferation over a wide range of stimulatory conditions and MAb concentrations.

Antibodies Against CTLA-4 Can Augment Proliferation in the Presence of Increased TCR Stimulation

The variation in the ability of 4F10 to augment proliferation...
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4F10 Fab Fragments Augment Proliferation in an Allogeneic MLR

The costimulatory function of anti-murine CD28 MAb in an allogeneic MLR requires MAb cross-linking on FcR-bearing APC or plastic (T. L. W. et al., unpublished data). In fact, Fab fragments of anti-CD28 severely inhibit T cell proliferative responses (Figure 6A). This is consistent with the anti-CD28 Fab fragments blocking the costimulatory interaction of the CD28 receptor with its natural ligands B7-1, B7-2, or both. Therefore, in the present study, the functional effects of Fab fragments of the anti-CTLA-4 MAB were compared with whole immunoglobulin to distinguish between anti-CTLA-4 costimulation of T cell proliferation and the anti-CTLA-4 MAb preventing the down-regulation of T cell activation following the interaction of CTLA-4 with its natural ligands. Pure 4F10 Fab fragments were prepared and added to an allogeneic MLR. The Fab fragments significantly augmented T cell proliferation (4-fold above control Fab fragments of 16-10A1) (Figure 6C). The augmented proliferation was 50% of that observed using the whole MAb at the same molar concentration (Figures 6B and 6C). The difference between the whole Ab and Fab fragments may reflect a decreased avidity of the monoclonal Fab fragments. Most importantly, the augmentation of proliferation observed with the anti-CTLA-4 Fab fragments is distinct from the effects of adding anti-CD28 Fab fragments to the allogeneic MLR (Figures 6A and 6C). These results suggest that anti-CTLA-4 MAb and Fab fragments may augment the allogeneic MLR by inhibiting the interaction of CTLA-4 with its natural ligand. Thus, CTLA-4 may be involved in the down-regulation of T cell responses following the initial activation event (Figure 5).

Anti-CTLA-4 MAbS Inhibit T Cell Proliferation in the Presence of Anti-CD3 and Anti-CD28

To examine directly the inhibitory effects of anti-CTLA-4 MAbS, the 4F10 MAb was added to T cell cultures suboptimally stimulated with anti-CD3 MAb and maximally costimulated with anti-CD28 MAbS. Under these conditions, 4F10 inhibited proliferation of purified B6 and 2C TCR transgenic T cells to anti-CD3 and anti-CD28 in the presence of syngeneic APC (Figures 7A and 7B, respectively). As observed previously (see Figures 4A, 5, and 6B), the 4F10 MAb under suboptimal TCR stimulation (in these experiments, 0.1 μg/ml anti-CD3) augments T cell proliferation in the absence of anti-CD28. However, the addition of the anti-CTLA-4 MAb to cultures containing anti-CD28 resulted in the inhibition of T cell proliferation even at concentrations as low as 3.5 μg/ml 4F10 MAb (Figure 5B). Interestingly, in contrast with the effects observed
using the whole anti-CTLA-4 MAb, Fab fragments of 4F10 did not inhibit proliferation (Figure 7C); in fact, the addition of Fab fragments augmented proliferation. The inhibition is not due to blocking FcR binding by the anti-CD3 or anti-CD28 MAb, because high concentrations (100 μg/ml in Figures 7A and 7B and 50 μg/ml in Figure 7C) of control hamster immunoglobulin had no inhibitory effect. In fact, in Figure 7A, the addition of the 4F10 MAb (100 μg/ml) to anti-CD3-stimulated (0.1 μg/ml) cultures augmented T cell proliferation. Thus, anti-CD3-mediated T cell activation that is FcR-dependent is not inhibited in this setting.

These results suggest that the addition of anti-CTLA-4 MAb to T cell stimulation assays has two distinct effects. Under conditions of maximal costimulation, the cross-linking form of 4F10 (whole immunoglobulin) inhibits T cell proliferation. In contrast, a soluble form of MAb (4F10 Fab fragments or whole MAb under conditions of limiting FcR cross-linking) results in augmentation (see Figure 6; Figure 7).

Discussion

During the past several years, it has become increasingly apparent that CD28 plays an important role in the regulation of T cell activation. Ligation of the CD28 receptor in the presence of TCR ligation results in IL-2 gene transcription and mRNA stabilization (June et al., 1990) and, under certain conditions, anti-CD28 MAb is capable of blocking energy induction in T cell clones (Harding et al., 1992). CTLA-4 is homologous to CD28, CTLA-4 shares approximately 30% amino acid identity with CD28 and, like CD28, binds the B7-1 and B7-2 molecules. However, while the role of CD28 in T cell activation is becoming more clear, the function of CTLA-4 is still uncertain. To develop a better understanding of the fundamental role of CTLA-4 in T cell function, a panel of MAb specific for murine CTLA-4 were developed and studied.

Several observations have been made with these reagents that confirm previous studies in the human model. First of all, CTLA-4 is only expressed on activated murine T lymphocytes. The kinetics of CTLA-4 expression on activated murine T cells closely parallels that previously reported for activated human T cells (Linsley et al., 1992a); CTLA-4 expression appears maximal at approximately 48 hr and becomes almost undetectable by 96 hr after T cell activation. Second, CTLA-4 is expressed on both CD4+ and CD8+ T cell subsets, although CD8+ T cells appear to express higher levels of CTLA-4 during the course of activation. Whether this differential level of expression relates to differential functions of the two subsets remains to be determined. Third, both TCR ligation and CD28 receptor ligation are important in the regulation of CTLA-4 expression. CTLA-4 expression is CD28-dependent; inasmuch as activated T cells with anti-CD3 alone did not up-regulate CTLA-4. The defect in the T cells from these mice may be due, in part, to a lack of IL-2 production, because the addition of IL-2 reconstituted CTLA-4 expression. However, other CD28-dependent costimulatory events are likely to be involved, given that CTLA-4 expression on the CD28-deficient activated T cells did not reach wild-type levels, even in the presence of exogenous IL-2.

In addition, several observations have been made with the MAb s that suggest that the expression and biological function of CTLA-4 may be more complicated than previously suggested. It appears that the human and murine CTLA-4 molecule may exist in multiple forms on the cell surface. Although the majority of CTLA-4 on activated murine T cells exists as a disulfide-linked dimer composed of two 33–37 kDa monomeric units, a nonsulf苜dine-linked, perhaps monomeric, form also exists. Previous studies have suggested that alternative forms of CTLA-4 may exist on human cells as well. Anti-CTLA-4 antisera was shown to immunoprecipitate a nonsulf苜dine-linked form of CTLA-4 from activated human PBL (Lindsten et al., 1993), whereas, an anti-human CTLA-4 MAb was shown to immunoprecipitate predominantly a disulfide-linked dimer of...
CTLA-4 from transfectants. These discrepant results may represent differences in the serological reagents used. In parallel studies, immunoprecipitation of CTLA-4 from CTLA-4GPI-transfected CHO cells yields three different patterns of immunoprecipitation depending on the Mab used to immunoprecipitate the glycoprotein (data not shown). One set of Mabs, including 4F10-12, precipitated predominantly the higher molecular mass disulfide-linked form; others, including 4F10 and 1B8, precipitated both the disulfide-linked form and the lower molecular mass form; while the remaining Mabs precipitate predominantly the monodisulfide-linked form. Thus, the studies with the CHO transfectants suggest that the pattern of CTLA-4 immunoprecipitation depends on the antibody used for immunoprecipitation. In fact, the presence of both a monodisulfide- and disulfide-linked form of CTLA-4 on the cell surface of activated murine T cells is not unprecedented; CD26 has also been demonstrated to exist in both a dimeric and monomeric form on T lymphocytes (Lesslauer et al., 1988). Finally, despite the homology between CD28 and CTLA-4, these molecules do not form heterodimers on activated murine T cells that express both glycoproteins, since the electrophoretic mobility of CTLA-4 dimers immunoprecipitated from activated T cells isolated from CD28-deficient mice is identical to the patterns seen from B6 mice.

Most importantly, the present studies examined the role of CTLA-4 in the regulation of T cell function. Anti-CTLA-4 MAb augmented T cell proliferative responses to alloantigen. However, rather than delivering a positive signal through the CTLA-4 receptor, the anti-CTLA-4 MAb appears to block transduction of a negative signal. First, Fab fragments of the 4F10 MAb augmented the allogeneic MLR. The inability of Fab fragments to be cross-linked by FcR on the allogeneic APCs argues strongly against initiation of a costimulatory signal mediated by CD28. The activated T cells were given suboptimal signal 1 stimulation with anti-CD3 but costimulated optimally using anti-CD28 MAb, the whole 4F10 but not the Fab fragments of 4F10 inhibited T cell proliferation. This down-regulation of T cell proliferation required both increased costimulatory signals and optimal cross-linking of the MAb. Thus, our data suggests that rather than playing an analogous role to CD28, CTLA-4 may be an antagonist of T cell function. In this model, resting T lymphocytes first encounter MHC–antigen complexes in the presence of a costimulatory signal mediated by CD28. The activated T cells produce IL-2 to drive T cell proliferation and up-regulate CTLA-4. At the time point of maximal CTLA-4 expression, CD28 function is diminished, since signaling through CD28, as evidenced by Ca²⁺ flux upon CD28 cross-linking, is depressed (Linsley et al., 1993). Thus, CTLA-4 can preferentially ligate with its counterreceptor, either B7-1 or B7-2, resulting in the transmission of a down-regulatory signal.

Although a number of cell surface molecules are involved in the costimulation or augmentation of T cell activation, molecules whose function it is to dampen or down-regulate T cell activation events to control T cell reactivity have not been extensively examined. CTLA-4 may represent one such molecule. The monoclonal reagents described here will be integral to study the cellular and biochemical behavioral consequences of CTLA-4 regulation of T cell activation in both in vitro and in vivo models of T cell immune response.

**Experimental Procedures**

**Animals**

Male DBA2, BALB/c, and C57BL/6 (B6) mice (6–12-week-old) were purchased from Frederick Cancer Research Institute Laboratories and housed in a specific-pathogen-free rodent facility at the University of Chicago. CD82 gene disrupted (CD82−/−) mice (4–16 weeks of age) were bred in the University of Chicago specific-pathogen free mouse facility. CD82 gene expression was disrupted in these animals through the replacement of exon 2 of the CD82 gene with a neo gene sequence as previously described (Shahinian et al., 1999). 2C TCR transgenic mice were developed as previously described. These animals were bred in University of Chicago specific-pathogen free mouse facility and used at 6–16 weeks of age. The 2C TCR transgenic mouse was generated using the 2C TCR clone, which has a TCR reactive with the class I MHC molecule L3T4. The presence of the transgenic TCR on the CD82 T cells in these mice is determined using the 1B2 anticonstitutive MAb (Sha et al., 1988).

**CTLA-4GPI Construct**

Stable transfection of CD28-cDNA expression constructs into commonly used cells such as CHO and NIH 3T3 does not lead to expression of cell surface CTLA-4 protein, as judged by the failure to bind B7-immunoglobulin (G. J. F., unpublished data). CTLA-4 was successfully expressed by linking the extracellular domain of CTLA-4 to a GPI anchor. The extracellular domain of CTLA-4 was amplified from a murine CTLA-4 cDNA by polymerase chain reaction using as sense primer, GAGTAAGCCGGCCACCACCTACGTGTTCTGGGA, containing a HindIII site, a strong start site, and the first 15 nt of the CTLA-4 coding sequence, an antisense primer, CACCGAATTCGACCACAAGCTACGTTACTGCTAGT-CAGAATTCGACCACAAGCTACGTTACTGCTAGT, containing the last 19 nt of the CTLA-4 extracellular domain and an AflII site. Polymerase chain reaction conditions were 94°C for 1 min, 45°C for 1 min, 72°C for 10 min, followed by 35 cycles followed by one cycle of 72°C for 10 min. The polymerase chain reaction product was digested with HindIII and AflII gel purified, and ligated into a HindIII- and AflII-digested pCDM8 vector containing the GPI anchor of human CD58 (Staunton et al., 1992; provided by Dr. D. Staunton, Center for Blood Research, Boston, Massachusetts). Plasmid containing the CTLA-4-GPI insert were transiently transfected into COS cells and strongly expressed cell surface CTLA-4, as judged by binding of B7-immunoglobulin fusion protein (G. J. F., data not shown).

**Antibodies and Cell Lines**

The P815 mastocytoma line, transfected with murine B7-1 was provided by L. Lanier (DNAx, Palo Alto, California) (Azuma et al., 1992). CTLA-4GPI-transfected CHO cells were generated using the CTLA-4GPI construct. 145-2C11 (anti-CD28) (Lee et al., 1987), UC3-10D4 (anti-V2) (Houlihan et al., 1988), 2.4G2 (rat anti-mouse Fc receptor) (Uehleke, 1979), 1B2 (anti-2C TCR) (Kantz et al., 1984), J11D (anti-heat-stable antigen) (Bruce et al., 1981), and the anti-I-Aβ MAb B25.5-3 and 25.5-17 (Ozato and Sachs, 1981) were prepared in our laboratory. (anti-Vα2) (Houlihan et al., 1988), 2.4G2 (rat anti-mouse Fc receptor) (Uehleke, 1979), 1B2 (anti-2C TCR) (Kantz et al., 1984), J11D (anti-MAG 16-10A1) (Rath-Wolf et al., 1992) and anti-CD28 Fab fragments were provided by Repligen Corporation (Cambridge, Massachusetts). Purified GL4 (anti-Vδ4) was provided by L. Lefrancois (University of Connecticut, Farmington, Connecticut). Both GL4 and purified hamster immunoglobulin (purchased from Cappel Research Products, Durham, North Carolina) were used as negative control hamster antibodies in in vitro cultures. Biotin-coupled anti-CD4 MAb (RM-4-1) Fluorescein isothiocyanate (FITC)-coupled anti-γ TCR (GL3) and phytohaemagglutinin (PHA) were used as positive controls for T cell proliferation.
coethrin-coupled anti-Thy1.2 (S3-2-I) were purchased from Phar-Mingen (San Diego, California); biotin-coupled anti-C0E (S3-6.7) was purchased from Becton-Dickinson (Mountain View, California). FITC-coupled 145-2C11 was provided by Boehringer Mannheim (Indianapolis, Indiana). Phycoethrin-coupled streptavidin was purchased from Southern Biotechnology (Birmingham, Alabama).

Production of Anti-Marine CTLA-4 mAbs

Anti-marine CTLA-4 antibodies were generated by immunizing Arme- nian hamsters with the extracellular portion of murine CTLA-4 fused to a murine IgG1 (mCTLA-4g; Wallace et al., 1994) by a protocol described previously (Leo et al., 1987). Subclones were generated by limiting dilution and tested for reactivity to murine CTLA-4g by an enzyme-linked immunosorbent assay. Subclones reactive to IgG1 Fc were eliminated by testing for cross-reactivity on a murine IgG1 MAb, 25.9.17, in an enzyme-linked immunosorbent assay.

T-Cell Activation Cultures

Splenocytes were harvested, mixed, and spleen cell suspensions were depleted of erythrocytes with ammonium chloride-potassium lysing buffer. Cells were cultured in DMEM containing 10% fetal calf serum, in the presence of 10 μg/ml soluble 145-2C11 in the presence or ab- sence of 50 U/ml IL-2 as indicated. For allogeneic reactions, T cells were activated with PB15–87-1 stimulator cells that had been treated with 50 μg/ml mitomycin C for 2–3 h at 37°C and washed 3 times in complete media before use. Responder LN or splenic T cells (2 × 10⁶) were cultured with an equal number of PB15–87-1 transfectants in 2 ml media per well over a 96 hr culture period.

Flow Cytometric Analysis

Cells to be examined were washed in FACS buffer (0.1% bovine serum albumin and 0.01% sodium azide in 1 x phosphate-buffered saline [PBS] pH 7.4) and incubated with anti-ICR MAb, 2.43, prior to staining. Directly FITC-coupled reagents and biotin-coupled reagents were added; incubated for 30 min at 4°C, washed, and incubated with phycoethrin-coupled streptavidin for 15 min at room temperature. Two- color flow cytometry was performed using a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, California) and analyzed using the Lysis II software packages. Logarithmi- cally-amplified fluorescence data were collected on 1 x 10⁶ viable cells as determined by forward scatter intensity and exclusion of dead cells based on staining with propidium iodide.

2D Polyacrylamide Gel Electrophoresis

Bulk CD28 (+) or CD86 spleen cell cultures were stimulated as de- scribed above for 72 hr, harvested, passed over Ficoll-Hypaque, washed extensively in phosphate-buffered saline (pH 7.4), and surface-labeled with ImmunoPure NHS-LC-biotin (Pierce, Rockford, Illi- nois) for 30 min at 14°C. Cells were lysed in 0.5% NP40 lysing buffer containing 50 mM Tris, 50 mM NaCl, 5 mM EDTA, 0.1% Nonidet (polyoxyethylene) glycol 900 (NP-40), 0.1% sodium deoxycholate, and 1 μg/ml pepstatin at 1 hr 4°C. Lysate preclearing was performed using hyperimmune rabbit sera and a control hamster antibody (UCS-164A) culture supernatant in the presence of protein A-Sepharose beads (Pharmacia, Piscataway, New Jersey). MAb's used for immunocep- tization were purified from tissue culture supernatant using protein A-Sepharose (Pharmacia) or protein G-Sepharose (Pharmacia) gel electrophoresis tube gels (10%) were electrophoresed under nonreducing conditions in the first dimension, transferred to a second 12.5% slab gel, and electrophoe- rezed under reducing conditions in the second dimension. Proteins were transferred into Immoblot (Schleicher and Schuell, Keene, New Hampshire) and blots were developed using a streptavidin–horse- radish peroxidase conjugate (Amersham Corporation, Arlington Heights, Illinois) in combination with the chemiluminescent LumiGlo substrate (Kirkegaard Perry Labs, Gaithersburg, Maryland).

T Cell Purification

B6 or 2C TCR transgenic lymph node cells were enriched for T cells by passage over nylon wool columns. Cells positive for MHC class II molecules were further depleted using a mixture of anti-heat-stable antigen (AHA) and anti-Thy1.2 (S3-2-I) antibodies as described elsewhere (Pel-Freyz, Brown Deer, Wisconsin). T cell purity was evaluated by flow cytometry using anti-CD3 MAbs (145-2C11). In all cases, T cells used in these assays were >95% CD3⁺.

Allogeneic MLR

Purified T cells were plated out at either 4 x 10⁶ cells/well for B6 T cells and at 2 x 10⁶ cells/well for 2C TCR transgenic T cells. γ-irra- diated (2000 rads) erythrocyte-depleted allogeneic stimulator cells, prepared from DBA2 or BALB/c spleens, were used at indicated num- ber per well in the B6 T and transgenic T cell assays. After 96 hr of incubation at 37°C, individual wells were pulsed with 1 μCi/well [3H]thymidine for the last 12–16 hr of culture. Counts are represented as the mean cpm of triplicate wells.

Allogeneic MLR in the Presence of L⁰-Binding Peptide

Allogeneic MLR assays were set up as described above, using the 2C TCR transgenic T cells as responders and 1.25 x 10⁶ irradiated BALB/c splenocyte feeders pulsed with indicated concentrations of L⁰-binding peptide, LPWP[DFD]L (described by Udaka et al., 1992), concentrations for 1 hr at 37°C before purified T cells were added. The L⁰ peptide was prepared by the University of Chicago peptide synthesis facility.

Preparation of Fab Fragments

4F10 and 16-10A1 MAb's in purified form were cleaved into Fab frag- ments using papain-immobilized agarose beads (Sigma, St. Louis, Missouri) for 72 hr at 37°C. Following fragmentation, the reaction mix- ture was passed over a protein A-Sepharose column to remove the remaining whole MAbs and Fc fragments. Fab fragments (50,000 MW) were further purified by subsequent filtration through Centricon100 filters to remove residual Fc fragments or whole MAbs, respectively (Amicon Incorporated, Beverly, Massachusetts). The pu- rity of the Fab fragments was confirmed by nonreducing SDS-polyacrylamide gel electrophoresis. The avidity of the Fab fragments was tested by comparing the ability of whole 4F10 MAb and Fab fragments to block the binding of FITC-coupled 4F10 MAb to transfected CHO cells expressing a GFP-linked form of the CTLA-4 molecule.

Anti-CD3-Induced Proliferation Assays

Purified B6 or 2C TCR transgenic T cells (2 x 10⁶) were cultured in the presence of 0.1 μg/ml anti-CD3, anti-CD28 MAb, PV-1 (1:1000 dilution of ascites), and 1 x 10⁶ syngeneic irradiated (2000 rads) B6 splenocytes. Counts are represented as the mean cpm of triplicate wells harvested at 72 hr following a 16 hr pulse with 1 μCi/well [3H]thym-idine.

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