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CTLA-4 Can Function as a Negative Regulator of T Cell Activation

Theresa L. Walunas, Deborah J. Lenschow, Christina Y. Bakker, Peter S. Linsley, Gordon J. Freeman, Jonathan M. Green, Craig B. Thompson, and Jeffrey A. Bluestone

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 (B70) 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, MYPPPY, 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-4ig. In fact, binding studies have demonstrated that CTLA-4ig has a 10- to 20-fold higher affinity for B7 than a similarly derived fusion protein with the extracellular domain of CD28 (Linsley et al., 1991). CTLA-4ig is also capable of blocking in vitro proliferation in assays of alloseactivity (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-4ig 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 mRNAs 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 MAb 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-4ig fusion protein. Candidate hybridomas were screened by using the CTLA-4ig molecule as a capture substrate for hybridoma supernatants in an enzyme-linked immunosorbent assay. The specificity of three MAbs, UC10-1B8, UC10-489-12, and UC10-4F10-11 (4F10) were confirmed on CHO cells transfected with a glycoporphosphatidylinositol (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 (Damle 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 MAbs 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 (P815/B7-1). Up-regulation of CTLA-4 on clonotype-positive T cells, stimulated with the H-2Ld allodonor, followed a time course similar to that previously observed with anti-CD3 (Figure 2B). Maximum 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 nominal antigen 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 Ficol 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 non-disulfide-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 non-disulfide-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
Figure 4. Anti-CTLA-4 MAb Can Augment Proliferation in an Allogeneic MLR.
(A) Purified 2C T cells (2 × 10⁵ cells/well) were cultured with the indicated numbers of allogeneic y-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 hr following the initiation of culture. Data is representative of three experiments.
(B) Purified 2C TCR transgenic T cells (2 × 10⁵/well) were cultured in the presence of 5 × 10⁵ y-irradiated (2000 rads) 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 × 10⁵ cells/well) were cultured with 1 × 10⁶ allogeneic y-irradiated DBA/2 splenocytes in the presence of either negative control hamster MAb, GL4 (190 μg/ml) or the indicated concentration of 4F10. Assays were harvested 96 hr following initiation of culture. Data is representative of five experiments.

homology shared between the two molecules. However, the data shown here provide no evidence for this association. First, the molecular mass of murine CD28 monomers is approximately 45 kDa, while CTLA-4 monomers appear to migrate between 33–37 kDa. Second, the pattern of CTLA-4 molecule expression is similar if not identical when activated T cells from normal and CD28 (−/−) mice were compared (Figure 3). Therefore, CTLA-4 does not need to heterodimerize with CD28 to be expressed on the surface of activated murine T cells. However, this does not preclude the fact that CTLA-4 and CD28 may heterodimerize under activation conditions not explored in this study.

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 response. 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 MAba (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 MAba. 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 MAba 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 MAba were able to enhance 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
**Figure 6.** 4F10 and 4F10 Fab Fragments Can Augment Proliferation in an Allogeneic MLR

(A) Purified B6 T cells (4 x 10^5 cells/well) were cultured with 4 x 10^6 allogeneic irradiated BALB/c splenocytes. Control hamster immunoglobulin (Cl Ig) and anti-CD28 Fab fragments (anti-CD28 Fab) are present at 100 μg/ml final concentration. SEM of Cl Ig = 192.2 and of anti-CD28 Fab = 956.

(B) Purified B6 T cells (4 x 10^5) were cultured in the presence of 5 x 10^5 irradiated (2,000 rad) BALB/c splenocytes. Control hamster immunoglobulin (Cl Ig) and 4F10 are present at a final concentration of 50 μg/ml. SEM of Cl Ig = 2830 and of 4F10 = 6782.

(C) Control hamster Fab fragments from anti-B7-1 MAb, 16-10A1, and 4F10 Fab fragments (4F10 Fab) are present at a final concentration of 16.7 μg/ml. SEM of Cl Fab = 1100 and of 4F10 Fab = 4065. Whole MAb reagents and Fab reagents were compared here at equimolar concentrations. Control reagents are represented by open bars. Anti-CD28 Fab fragments, 4F10 and 4F10 Fab fragments are represented by the shaded bars. This figure is representative of three separate experiments.

may reflect the degree of initial activation mediated by TCR engagement with relevant antigen. Therefore, an antigen dose titration was performed (Figure 5). The 2C TCR is reactive to a known peptide (LSPFPFDL) in the context of the MHC class I molecule, H-2L^d (Udaka et al., 1992). Addition of this peptide to allogeneic H-2L^d-expressing stimulator cells increases the number of H-2L^d-peptide complexes on the cell surface of the H-2L^d* stimulator cells (Smith et al., 1992), such that there is increased ligation of the TCR on the surface of the transgenic T cells without increasing the level of costimulatory molecules available to the T cells. Under these conditions, 4F10 augmented proliferation when peptide was added over a range of concentrations from 1 μg/ml to 0.01 μg/ml. As seen in the dose titration of allogeneic stimulators (see Figure 4A), the greatest augmentation was observed under suboptimal TCR stimulation. However, even at high concentrations of peptide (i.e., 1 μg/ml), anti-CTLA-4 MAb was able to augment T cell proliferation significantly in the allogeneic MLR as compared with control hamster immunoglobulin (Figure 5).

### 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 inhibited 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 monovalent Fab fragments. Most importantly, the augmentation of proliferation observed with the anti-CTLA-4 Fab fragments is distinctly different 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 MAb. 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 8.3 μg/ml 4F10 MAb (see 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 Mabs, 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 these 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 are 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 was 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 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 non-disulfide-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 non-disulfide-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 yield 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 18A, 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 nondisulfide- 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 (Leslaur et al., 1988). Finally, despite the homology between CD28 and CTLA-4, these molecules do not form homodimers 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 appear 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 CTLA-4. In fact, similar studies performed using anti-CD28 MAb showed that costimulation could only be observed using whole Ab. Fab fragments of anti-CD28 profoundly inhibited the allogeneic MLR. Furthermore, when 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 (Linsey 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 DBA/2, BALB/c, and C57BL/6 (B6) mice (6- to 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. CD28 gene disrupted (CD28--/--) mice (4-16 weeks of age) were bred in the University of Chicago specific pathogen free mouse facility. CD28 gene expression was disrupted in these animals through the replacement of exon 2 of the CD28 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 L. The presence of the transgenic TCR on the CD8+ T cells in these mice is determined using the 1B2 anti-clonotypic MAb (Sha et al., 1988).

**CTLA-4GPI Construct**

Stable transfection of CTLA-4 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, GAGTAAAGCTTGCGGCCAACCATGGTCTTGAGTG, containing a HindIII site, a strong start site, and the first 15 nt of the CTLA-4 coding sequence, an antisense primer, GAGAATTTCTAGACTAGCTATTGTCAGAATCTGGCGGACGCGT, 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, 42°C for 1 min, 72°C for 1 min for 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 CD8 (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., unpublished 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. 1A1C10-11 (anti-CD28) (Lee et al., 1987), UC3-10D4 (anti-V2) (Houlden et al., 1989), 2.432 (rat anti-mouse Fe receptor) (Unkeless, 1979), 1B2 (anti-2C TCR) (Kranz et al., 1994), J11d (anti-heat-stable antigen) (Bruce et al., 1981), and the anti-II-4 MAb 25.9.3 and 25.9.17 (Czabo and Sachs, 1981) were prepared in our laboratory, (anti-V2) (Houlden et al., 1989), 2.432 (rat anti-mouse Fe receptor) (Unkeless, 1979), 1B2 (anti-2C TCR) (Kranz et al., 1994), J11d (anti-MAA (16-10A1) (Rahn-Wool et al., 1992) and anti-CD28 Fab fragments were provided by Repligen Corporation (Cambridge, Massachusetts). Purified GL4 (anti-V54) was provided by L. Letfairanson (University of Connecticut, Farmington, Connecticut). Both GL4 and purified human immunoglobulin (purchased from Cappel Research Products, Durham, North Carolina) were used as negative control hamster antibodies in in vitro cultures. Biotin-conjugated anti-CD4 MAb (RM4-4), Fluorescein isothiocyanate (FITC)-coupled anti-γ TCR (GL3) and phy-
coerythrin-coupled anti-Thy1.2 (S3-2-1) were purchased from Pharmingen (San Diego, California); biotin-coupled anti-CDE (S3-6.7) was purchased from Becton-Dickinson (Mountain View, California). FITC-coupled 145-2C11 was provided by Boehringer Mannheim (Indianapolis, Indiana). Phycoerythrin-coupled streptavidin was purchased from Southern Biotechnology (Birmingham, Alabama).

Production of Anti-Marine CTLA-4 MAb

Anti-marine CTLA-4 antibodies were generated by immunizing Arme-

nian hamsters with the extracellular portion of murine CTLA-4 fused to a murine IgG (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-4 g by an enzyme-linked immunosorbent assay. Subclones reactive to IgG Fc were eliminated by testing for cross-reactivity on a murine IgG2b, 25-9,17-1, in an enzyme-linked immunosorbent assay.

T Cell Activation Cultures

Spleens were harvested, minced, 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 absence of 50 U/ml IL-2 as indicated. For allelogenic 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 L929 or spleen T cells (2 × 10⁵) were cultured with an equal number of PB15-87-1 transfectants in 2 ml media wells 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 [pH 7.4]) and incubated with anti-FcR 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 phycoerythrin-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 10⁹ 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 B6 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-IC-biotin (Pierce, Rockford, Illi-
nois) for 30 min at 14°C. Cells were lysed in 0.5% NP40 lysis buffer containing 50 mM Tris, 50 mM NaCl, 5 mM EDTA, 0.1% Tween 80, 1% SDS, 0.1% sodium dodecyl sulfate, and 1 μg/ml pepstatin at 1 h at 4°C. Lysate preclearing was performed by hyperimmune rabbit sera and a control hamster antiserum (UCS-16A9) culture supernatant in the presence of protein A-Sepharose beads (Pharmacia, Piscataway, New Jersey). MAb used for immunoprecipita-
tion were purified from tissue culture supernatant using protein A-Sepharose 2D-polyacrylamide gel electrophoresis tube gels (10%) were electroeluted under nonreducing conditions in the first dimension, transferred to a second 12.5% slab gel, and electropho-
reased under reducing conditions in the second dimension. Proteins were transferred into nicoct笙ella (Schleicher and Schuell). Keene, New Hampshire) and blots were developed using a streptavidin–horserad-
ish peroxidase conjugate (Amersham Corporation, Arlington Heights, Illinois) in combination with the chemiluminescent LumiGlo substrate (Kirkegaard Penny 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 (HSA) and anti-Thy1.1 (25-9-1) culture supernatants plus rabbit complement (Pel-Freeze, Brown Deer, Wisconsin). T cell purity was evaluated by flow cytometry using anti-CD23 MAb (145-2C11), in all cases, T cells used in these assay were >95% CD3+.

Allogeneic MLR

Purified T cells were plated out at either 4 x 10⁵ cells/well for B6 T cells and at 2 x 10⁵ cells/well for 2C TCR transgenic T cells. γ-irradiated (2000 rads) erythrocyte-depleted allogeneic stimulator cells, prepared from DBA/2 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 Lα-Binding Peptide

Allogeneic MLR assays were set up as described above, using the 2C TCR transgenic T cells as responders and 2.5 x 10⁵ irradiated BALB/c splenocyte feeders pulsed with indicated concentrations and Lα-binding peptide, LQPPFDL (described by Udaka et al., 1992), concentrations for 1 hr at 37°C. Following fragmentation, the reaction mix-
ture was passed over a protein A-Sepharose column to remove the remaining whole MAb and Fc fragments. Fab fragments (50,000 MW) were further purified by successive filtration through Centricon30 and Centicon100 filters to remove residual Fc fragments or whole MAb, respectively (Amicon Incorporated, Beverly, Massachusetts). The pur-
ity of the Fab fragments was confirmed by nonreducing SDS–poly-
acrylamide 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 transfect CHO cells expressing a GP-I-linked form of the CTLA-4 molecule.

Anti-CD3-Induced Proliferation Assays

Purified B6 or 2C TCR transgenic T cells (2 x 10⁷) 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 10⁵ 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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References


liferative responses and cytotoxicity mediated by small, resting T lympho-


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CTL-4 Function in T Cell Activation


