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CTLA-4-Mediated Inhibition of Early Events of T Cell Proliferation

Monika C. Brunner,* Cynthia A. Chambers,* Francis Ka-Ming Chan,* Jeff Hanke,† Astar Winoto,* and James P. Allison*‡

CTLA-4 engagement by mAbs inhibits, while CD28 enhances, IL-2 production and proliferation upon T cell activation. Here, we have analyzed the mechanisms involved in CTLA-4-mediated inhibition of T cell activation of naive CD4⁺ T cells using Ab cross-linking. CTLA-4 ligation inhibited CD3/CD28-induced IL-2 mRNA accumulation by inhibiting IL-2 transcription, which appears to be mediated in part through decreasing NF-AT accumulation in the nuclei. However, CTLA-4 ligation did not appear to affect the CD28-mediated stabilization of IL-2 mRNA. Further, CTLA-4 engagement inhibited progression through the cell cycle by inhibiting the production of cyclin D3, cyclin-dependent kinase (cdk)4, and cdk6 when the T cells were stimulated with anti-CD3/CD28 and with anti-CD3 alone. These results indicate that CTLA-4 signaling inhibits events early in T cell activation both at IL-2 transcription and at the level of IL-2-independent events of the cell cycle, and does not simply oppose CD28-mediated costimulation. 


Optimal activation and differentiation of naive CD4⁺ T cells to cytokine-producing effector cells requires engagement of the TCR/CD3 complex and the costimulatory molecule CD28. The function of CD28 homologue CTLA-4 has been more controversial. Although CD28 and CTLA-4 share the same ligands, namely B7.1 and B7.2, CTLA-4 binds with an affinity that is 10- to 20-fold higher than that of CD28 (1, 2) and has a unique expression pattern (3, 4). With the exception of two reports (5, 6), recent evidence indicates that CTLA-4 plays an inhibitory rather than a positive costimulatory role in regulating T cell responses. This idea was initially suggested by the observation that soluble anti-CTLA-4 Abs, both intact and Fab fragments, augment T cell responses in vitro (7, 8). Blocking CTLA-4/B7 interaction in vivo also increases T cell responses to antigenic challenges (9, 10) and enhances T cell-mediated tumor rejection, parasite clearing (11–13), and autoimmune disease progression (reviewed in Ref. 14, and Ref. 15). In addition, CTLA-4 cross-linking in conjunction with anti-CD3/CD28 cross-linking results in inhibition of T cell proliferation and IL-2 secretion by murine (7, 8, 16, 17) and human T cells (18). The lack of a positive costimulatory role for CTLA-4 was indicated by the absence of B7-dependent responses in CD28-deficient mice (19). Moreover, CTLA-4 blockade exacerbates graft-vs-host disease generated with grafts from CD28⁻/⁻ mice (20), and allograft and tumor rejection in CD28⁻/⁻ mice (21, 22). Finally, mice with a null mutation in CTLA-4 display a massive polyclonal T cell activation and expansion that results in a dramatic, fatal lymphoproliferative disorder (23, 24). The dramatic phenotype of these mice does not appear to be due to a defect in thymic development or selection, since both appear normal in the CTLA-4 null mice (25, 26). Rather, this activation is mediated by CD28-dependent activation (27, 28) of peripheral CD4⁺ T cells (27). Together, these observations provide a compelling case for an inhibitory role for CTLA-4 in the regulation of T cell responses.

The stage at which CTLA-4 exerts its inhibitory effects has not been clearly defined. CD28 is constitutively expressed on T cells, whereas CTLA-4 is expressed at low, undetectable levels on naive T cells and is induced upon T cell activation (7, 8, 29–33). The fact that CTLA-4 is not readily detectable on resting cells and is not expressed at maximal amounts on the cell surface until about 48 h after T cell activation has led to the notion that its function is to regulate ongoing T cell responses (34). However, CTLA-4 mRNA is already detectable within 1 h after activation in previously activated human T cells (30) and is detectable by PCR in samples prepared from purified naive murine CD4⁺ T cells (M.C.B. and J.P.A., unpublished observations). Also, CTLA-4 has a unique intracellular localization, with the majority of the CTLA-4 protein being retained inside the activated T cells (3, 4, 33). These observations suggest that CTLA-4 is present at physiologically significant levels and may regulate T cell responses much earlier than indicated by cell surface expression.

Although it was initially reported that CTLA-4 cross-linking induced apoptosis (35), other studies showed that CTLA-4 engagement during T cell activation prevents progression through the cell cycle without causing apoptosis (16, 17). Rather, it appears that CTLA-4 engagement inhibits early T cell activation events, including the induction of IL-2R α-chain and CD69 expression, the increase in T cell volume, and the production of IL-2 (16–18). The mechanisms involved in the inhibition of these responses are unknown. Optimal IL-2 secretion requires CD28-mediated costimulation (reviewed in Ref. 36). Two mechanisms have been reported to account for the enhanced IL-2 mRNA accumulation upon costimulation: prolongation of the IL-2 mRNA half-life (37, 38) and...
transcriptional activation of the IL-2 gene (39), perhaps as a result of increased levels of the transcription factors NF-AT and AP-1 in the nucleus (40). Cell cycle progression is regulated by a series of cyclins, cyclin kinases, and inhibitors (reviewed in Ref. 41). In T cells, the G1/G1 to S transition is mediated early in G1, mostly through a combination of cyclin D2/D3 with cyclin-dependent kinase (cdk)4/6/cdk6 and cyclin E/cdk2 in late G1 (see Refs. 56, 57, 62–64). How CTLA-4 cross-linking affects these pathways at the molecular levels is not known.

In this study, we examine the mechanisms involved in CTLA-4-mediated inhibition of T cell activation. Using Ab-coated microspheres, we demonstrate that CTLA-4 cross-linking inhibits anti-CD3/CD28 Ab-induced IL-2 mRNA accumulation. CTLA-4 engagement inhibits luciferase production by CD4+ T cells from mice bearing a luciferase reporter gene under the control of the IL-2 promoter, indicating that IL-2 gene transcription is inhibited. This effect appears to be at least partially due to the inhibition of NF-AT nuclear translocation. Conversely, the CD28-mediated IL-2 mRNA stabilization does not appear to be affected by CTLA-4 cross-linking. CTLA-4 engagement also prevents progression through the cell cycle, by inhibiting the production of the cell cycle proteins cyclin D3 and cdk4, which are partially IL-2-dependent, and cdk6, which is IL-2-independent, and altering the degradation of the cell cycle inhibitor p27. Interestingly, CTLA-4-mediated effects on cell cycle proteins were observed when the cells were stimulated via CD3 alone, suggesting CTLA-4 can inhibit CD28-independent pathways in T cell activation.

Materials and Methods

Mice

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The IL-2-luc transgene was generated by inserting a 2160-bp genomic fragment from the human growth hormone gene (42) into pBlueScript SK− (−) (Stratagene, La Jolla, CA), and the firefly luciferase gene was cloned upstream of the hgh gene to yield vector pSKluxhgh. The promoter region of the murine IL-2 gene (positions −590 bp to +40 bp) (43) was PCR-amplified from murine genomic DNA (Clontech, Palo Alto, CA) and inserted upstream of the luciferase gene. The mouse γ-globulin gene matrix association region (positions 2849–3764; kindly provided by William Garrard, University of Texas Southwestern, Dallas, TX (44)) was inserted upstream of the IL-2 promoter to ensure stable integration into the genomic DNA. The transgene was excised from the final vector, purified, and inserted upstream of the IL-2 promoter to yield vector pSKluxhgh. The promoter region of the murine IL-2 gene (positions −590 bp to +40 bp) (43) was PCR-amplified from murine genomic DNA (Clontech, Palo Alto, CA) and inserted upstream of the luciferase gene. The mouse γ-globulin gene matrix association region (positions 2849–3764; kindly provided by William Garrard, University of Texas Southwestern, Dallas, TX (44)) was inserted upstream of the IL-2 promoter to ensure stable integration into the genomic DNA. The transgene was excised from the final vector, purified, and inserted upstream of the luciferase gene. The mouse γ-globulin gene matrix association region (positions 2849–3764; kindly provided by William Garrard, University of Texas Southwestern, Dallas, TX (44)) was inserted upstream of the IL-2 promoter to ensure stable integration into the genomic DNA.

Ab and reagents

RPMI 1640 (BioWhittaker, Walkersville, MD) was supplemented with 10% calf serum (HyClone, Logan, UT), 2% M-2 ME (Sigma), 2 mM L-glutamine, and 100 U/ml antibiotics (Life Technologies, Gaithersburg, MD).

The Abs used for activation were: anti-CD3 (hybridoma 500A2 (45)), anti-CD28 (hybridoma 37.N.51.1 (46)), anti-CD28 Ab (hybridoma 9H57) (48)), and anti-CD8 Abs (PharMingen, San Diego, CA) were used for flow cytometry. Anti-IL-2 and anti-IL-2-biotin Abs (PharMingen) were used for the ELISA. Sulfate polysylayte latex microspheres of 5 μm ± 0.1 μm mean diameters were obtained from Interfacial Dynamics (Portland, OR). Cyclosporin A (CSA) was a gift from Sandoz (East Hanover, NJ).

RNA preparation and quantitative RT-PCR

RNA samples were obtained from single-cell suspensions of CD4+ T cells (1 × 10^6 cells/sample) using guanidinium isothiocyanate by standard techniques. A total of 1–4 μg of RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Life Technologies/BRL) and oligo(dT) primer (Boehringer Mannheim, Indianapolis, IN). The cDNA equivalent of 5 ng RNA was amplified containing 250 nM each of dNTP (dATP, dCTP, dGTP, dTTP), 200 nM specific oligo primers (Cancer Research Lab, University of California Berkeley) and 1 U Taq Polymerase (AmpliTag; Perkin-Elmer/Cetus, Norwalk, CT) for 35 cycles: 1 min at 95°C, 2 min at 60°C, and 2 min at 72°C. PCR products were run on a 1.2% agarose gel by electrophoresis. Amplification products were analyzed for specificity by restriction analysis with two enzymes indicative of the expected amplified sequence. The primer sequences specific for β-actin and IL-2 were used as described (51). The DNA competitor fragments were obtained from the plasmid pMCQ, kindly provided by Dr. T. Blankenstein (Max-Delbrück Center for Molecular Medicine, Berlin, Germany) (52). Each cDNA aliquot was adjusted to contain an equal amount of β-actin (5 × 10^6 molecules) based on a comparison to the competitor DNA. Amplification with IL-2-specific primers was performed in the presence of serial dilutions (1:2) of competitor fragments, to estimate the amount required to achieve equal band intensities for both fragments (52, 53). For mRNA stabilization experiments, the cDNA was normalized by calculation to the same amounts of β-actin (5 × 10^6 molecules), and values were plotted as log_{10} molecules of competitor fragment.

Preparation of CD4+ T lymphocytes

Single cell suspensions were prepared from the lymph node cells. CD4+ T cells were isolated by treatment with complement, anti-MHC class II Abs, and anti-CD8 Abs, as described (17). Typically, the cell preparations were >96% CD4+ cells. In some experiments, CD4+ T cells were isolated by cell sorting from CD4+ cells enriched spleen and lymph node cells using an ELITE (Coulter Electronics, Hialeah, FL). The recovered cell population was >99% CD4+ T cells.

Activation of CD4+ T cells

Latex microspheres were coated as described (17). Briefly, 1 × 10^7 beads/ml were suspended in PBS with the indicated Abs and incubated for 1.5 h at 37°C, followed by washing with PBS and blocking with 10% FCS. Anti-CD3 (1 μg/ml), anti-CD28 (1.2 μg/ml), and anti-CTLA-4 (2 μg/ml) were added and control hamster anti-γ/δ (536) Ab was added to maintain a constant total Ab concentration of 5 μg/ml. T cells (1 × 10^6) were incubated in a ratio of 1:1 with beads in 96-well plates. Cultures were incubated for indicated lengths of time and pulsed with [3H]thymidine (1 μCi) 12 h before harvesting. Plates were harvested to glass filter mats and incorporation was measured using a gas-phase counter (Packard, Meriden, CT). For all of the experiments that required the cells to be collected at early time points of T cell activation, proliferation assays were performed in duplicate to ensure that beads performed appropriately. Ab-coated beads were also tested using CD4+ CTLA-4+ T cells (25), and the presence of anti-CTLA-4 Ab did not alter the proliferative response to CD3/28 cross-linking in these experiments.

Luciferase assay

Luciferase assays were performed according to the luminoimeter manufacturer’s instructions, as described (Luciferase Assay Guide Book, Analytical Luminescence Laboratory, 1992; Ref. 54). Cell extracts were prepared by lysing the cell pellets (3 × 10^6 cell/sample) in lysis buffer and, after 15 min at room temperature, were centrifuged for 5 min at 1000 × g to remove cell debris. The samples were divided into three 100-μl aliquots to which 0.2 μM enzyme coenzyme A, 300 μl luciferase assay buffer, and 100 μl 1 mM luciferin substrate were added. The luciferase activity was assayed using the Dynatech (Chantilly, VA) luminoimeter on integrated flash mode. Values presented are relative light units gained by integrating over 20-s minus light units of wells with buffer plus luciferin alone.

Measurement of lymphokine production

IL-2 in cell supernatants was detected by ELISA, as described (17). The standard curve was generated using rIL-2 (Boehringer Mannheim), and the level of detection was 2 ng/ml.
Western blot analysis

CD4+ primary T lymphocytes cells were pelleted and lysed in Triton buffer (150 mM NaCl, 10 mM Tris (pH 8.0), 1% Triton X-100, and 1 mM PMSF) for 10 min on ice. The lysates were spun down at 4°C for 5 min. The resulting supernatants were harvested, and protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA). Forty micrograms of each sample was loaded onto a 12% SDS-polyacrylamide gel and resolved electrophoretically. The gel was then transferred to nitrocellulose membrane and stained with Ponceau S to ensure equivalent loading and transfer. Western blot analysis was performed using 1 μg/ml of anti-cdk4, anti-cdk6, anti-cyclin D3, anti-p27 Abs (all from Santa Cruz Biotechnology, Santa Cruz, CA), or 1 μg of a polyclonal rabbit anti-p19 Ab (55). The bound Ab was detected by the appropriate HRP-conjugated secondary Ab. Blots were developed with the Renaissance Chemiluminescence Reagent (Dupont NEN, Wilmington, DE).

Gel mobility shift assays

Nuclear extracts of CD4+ T cells were prepared as reported (54). Based on the kinetics of cyclin D3 expression reported using human T cells (56, 57), the extracts were prepared 22 h after activation. The oligonucleotides for NF-AT (GTTGCCCAAAGAGGAAAATTTGTTTCATACAG) and AP-1 (CGCTTGATGACTCAGCCGGAA) were synthesized in the Microchemical Facility of the Cancer Research Laboratory (University of California). Binding reactions of the 32P-labeled probe (Amersham, Arlington Heights, IL) with 4 μg of nuclear extracts were performed at room temperature for 20 min. Binding of the radioactive probe was completely blocked by the addition of 20-fold excess nonradioabeled probe, whereas addition of a sequence from an irrelevant site (NBRE) did not alter the binding of the radioactive probes. Bound and unbound probes were resolved electrophoretically on a 4% native polyacrylamide gel. For NF-AT binding, the reaction mix contained 10 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM DTT, and 10% glycerol. For AP-1 binding, reaction mix contains 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 100 mM KCl, and 10% glycerol.

Results

CTLA-4 engagement inhibits accumulation of IL-2 mRNA in CD4+ T cells

CD4+ lymph node T cells (LNT) were stimulated with Ab-coated beads, and proliferation assays were performed as previously described (8, 17). As reported (17), CD4+ LNT proliferation and IL-2 secretion to suboptimal doses of anti-CD3 was augmented by CD28-mediated costimulation, and this response was inhibited by cross-linking CTLA-4 (data not shown). To detect early effects of CTLA-4 engagement on IL-2 transcription, IL-2 mRNA levels in CD4+ LNT cells stimulated under these conditions were assessed by competitive PCR. Competitor DNA fragments are included in the samples, and the same primers amplify both fragments, thus the amount of cDNA/sample can be quantitated by

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** CTLA-4 engagement inhibits IL-2 mRNA accumulation in CD4+ T cells upon activation. A, Resting CD4+ T cells were stimulated with anti-CD3, anti-CD3 and anti-CD28, anti-CD3 and anti-CD28 plus anti-CTLA-4, or a control Ab and analyzed for IL-2 mRNA expression by quantitative PCR at 7-h postactivation. Left, All cDNAs were equalized to 5 × 10^6 molecules of β-actin by coamplification of constant amounts of cDNA and 2-fold diluted control fragments. The arrow indicates the amplified competitor fragment. Right, Constant amounts of the calibrated cDNAs were coamplified with specific primers for IL-2 in the presence of 2-fold serially diluted competitor fragments starting with 1 × 10^2 molecules. The arrow indicates the amplified competitor fragment. B, Resting CD4+ T cells were stimulated with anti-CD3 (diamond), anti-CD3, and anti-CD28 (open circles), anti-CD3 and anti-CD28 plus anti-CTLA-4 (filled circles), or a control Ab (square) and analyzed for IL-2 mRNA expression by quantitative PCR at the times indicated. The lower detection limit was considered to be ~250 molecules of competitor fragments. Each time point has been repeated three to five times with similar results.
comparison to known amounts of the competitor fragment over a series of dilutions (52, 53). The product from the competitor fragment can be distinguished from the endogenous IL-2 mRNA by gel electrophoresis (Fig. 1) and by digestion with unique restriction enzymes. In the first step, the cDNA concentrations are normalized using β-actin as a standard. The cDNA concentration that was required to achieve equal band intensities for both the cDNA and the competitor fragments was determined by coamplifying serial dilutions of the competitor fragment and constant amounts of cDNA, thus ensuring that equal amounts of cDNA were compared in subsequent experiments. In the second step, the calibrated cDNAs were used for IL-2 cDNA amplification in the presence of serial dilutions of competitor fragments containing IL-2 primers-specific sequences (Fig. 1A).

As shown in Fig. 1, CD4+ T cells stimulated with beads coated with anti-CD3 alone accumulated very little IL-2 mRNA. Stimulation with anti-CD3 plus anti-CD28-coated beads resulted in rapid accumulation of IL-2 mRNA at levels up to 100-fold higher than stimulation with anti-CD3 alone. This occurred as early as 4 h after stimulation. Levels peaked at 10 h, and then declined (Fig. 1B). Interestingly, when T cells were stimulated by anti-CD3 plus CD28 in the presence of anti-CTLA-4, IL-2 mRNA accumulation was greatly inhibited over the entire time course. Similar to a recent report using human T cells (18), the early appearance of IL-2 mRNA was blocked by anti-CTLA-4 cross-linking, and, after more than 10 h, reached levels only <1% of those in control costimulated cultures.

**FIGURE 2.** IL-2 mRNA decay does not appear to be altered by CTLA-4 engagement after anti-CD3 plus anti-CD28 stimulation. Resting CD4+ T cells were precultured for 4 h with Ab-coated microspheres, as indicated before CsA addition. Cells were harvested for mRNA assay at the indicated time points after the CsA addition (time zero). Quantitative PCR was performed for IL-2 mRNA. All cDNAs were normalized to 5 × 10⁶ molecules of β-actin. Values are expressed as log₁₀ molecules of competitor fragments. One of two similar experiments is shown.

**FIGURE 3.** Luciferase induction is inhibited by CTLA-4 cross-linking in naive CD4+ T cells from a IL-2P-luc transgenic mouse. CD4+ T cells from IL-2P-luc mice were stimulated with Ab-coated microspheres as indicated. Cells were harvested at 4.5 h, lysed, and the supernatant was assayed for luciferase activity. Each point represents triplicates of 1 × 10⁶ cells. All results are represented as means ± SD. This is representative data from three experiments.

**CTLA-4 cross-linking does not appear to interfere with the stabilization of IL-2 mRNA by CD28**

One effect of CD28 costimulation is to stabilize the mRNA of a number of cytokine genes, including IL-2, all of which have an AU-rich sequence in the 3′ noncoding region (37, 38). Therefore, we sought to determine whether the inhibitory effect of CTLA-4 ligation on IL-2 mRNA accumulation was a result of mRNA destabilization (Fig. 2). To do this, CsA was added to the cultures 4 h after T cell stimulation with Ab-coated beads, and competitive RT-PCR was used to follow the IL-2 mRNA levels. As shown in Fig. 2, only minimal amounts of IL-2 mRNA were produced in anti-CD3-stimulated cells, and levels began to decrease rapidly 70 min after CsA addition. These results are comparable to that reported for a Th1 clone stimulated with plate-bound anti-CD3 (38).

In cultures activated with anti-CD3 and anti-CD28, there was a 500-fold increase in the absolute amount of IL-2 mRNA, and only a minor decay of IL-2 mRNA was detected following the addition of CsA. When T cells were stimulated with anti-CTLA-4 in conjunction with anti-CD3/28, the absolute amount of IL-2 mRNA was <1/50th of that observed with CD3 plus CD28 cross-linking alone (Fig. 2). However, the slope of the curves of the IL-2 mRNA levels appears similar, suggesting that the rates of decay are unchanged. Thus, CTLA-4 engagement does not appear to accelerate the decay of IL-2 mRNA and does not appear to reverse the stabilization mediated by CD28 cross-linking.

**Transcription initiation of IL-2 in freshly isolated CD4+ T cells is enhanced by CD28 and inhibited by CTLA-4 engagement**

To examine the effects of CTLA-4 ligation on the IL-2 promoter, we used a transgenic mouse bearing a luciferase reporter gene under the transcriptional regulation of the IL-2 promoter and enhancer (IL-2P-luc). The transgene behaved similarly to the endogenous IL-2 gene, as determined by examining the effects of PMA/ionomycin and anti-CD3/PMA on luciferase activity in CD4+ T cells from these mice. These stimuli led to the rapid induction of luciferase activity, and the effect was inhibited by CsA (data not shown). Proliferation and IL-2 production by CD4+ T cells from IL-2P-luc transgenic mice and control mice were similar (data not shown), indicating that the transgenic T cells behaved normally in response to polyclonal stimulation.

CD4+ lymph node T cells from IL-2P-luc mice were stimulated with the various combinations of anti-CD3-, anti-CD28-, and anti-CTLA-4-coated beads, and luciferase activity was assayed after 4.5 h. As shown in Fig. 3, minimal luciferase activity was induced by stimulation with anti-CD3 only, but this was enhanced >60-fold by CD28 costimulation, consistent with the data obtained by competitive PCR. As with endogenous IL-2 expression (data not shown), the induction of luciferase activity by anti-CD3 plus anti-CD28 was largely inhibited by CsA (Fig. 3). Cross-linking CTLA-4 during stimulation resulted in an almost complete (90%) inhibition of luciferase production by the T cells. These results indicate that CTLA-4 inhibits IL-2 production by altering the transcriptional regulation of the IL-2 promoter and that this occurs very early after T cell activation.
CTLA-4 engagement inhibits the accumulation of the transcription factor NF-AT, but not AP-1, in the nuclei of activated CD4\(^+\) T cells

Five NF-AT sites have been reported to be essential for full induction of the IL-2 promoter (reviewed in Ref. 58), four of which bind AP-1 in association with NF-AT. Upon activation, NF-AT and AP-1 translocate from the cytoplasm to the nucleus. Gel shift assays were performed to assess the effects of CTLA-4 engagement on the nuclear accumulation of NF-AT and AP-1. As shown in Fig. 4A, stimulation with anti-CD3 alone increased the accumulation of NF-AT in the nucleus over the basal levels, which was further augmented upon coligation of CD28. This increase paralleled enhancement of IL-2 mRNA transcription initiation (Fig. 3), IL-2 mRNA accumulation (Fig. 1), and ultimately, IL-2 production (data not shown, and Ref. 17). Coligation of CTLA-4 in conjunction with anti-CD3/28 inhibited NF-AT translocation to levels similar to those observed when T cells were stimulated with anti-CD3 alone (Fig. 4A). The induction of NF-AT was CsA-sensitive, regardless of the stimulation conditions (Fig. 4A). Since only the translocation of the factor to the nucleus is shown to be CsA-sensitive (59), only NF-AT that had translocated from the cytoplasm to the nuclear extracts was measured. In contrast, no obvious changes in the AP-1 translocation to the nuclear fraction upon CTLA-4 engagement could be detected in these assays (Fig. 4B). Levels of the Sp1 family binding to the GT-box element of the IL-2 promoter were also unaltered under all culture conditions (data not shown).

CTLA-4 cross-linking and expression of cyclin D3, cdk4, cdk6, p27, and p19

Entry into the S phase of the cell cycle is regulated by complex interactions between cyclin/cdkks and cell cycle inhibitors (reviewed in Ref. 41). Initial activation of cyclin D2/3 complexed to cdk4/6, followed by the induction of cyclin E/cdk2 activity are required for the entry into S phase. The p16 family of the cell cycle inhibitors (p16, p15, p18, and p19) can regulate the activity of cyclin D/cdk4/6, while p27 inhibits the kinase activity of cyclin E/cdk2. Stimulation of resting T cells through the TCR complex results in transcriptional activation of cyclin D2, D3, cdk4, and cdk6, as well as degradation of p27 (60, 61). Induction of cdk6, cdk4, and cyclins in normal T cells is partially dependent on IL-2 (56, 62, 63). In contrast, degradation of p27 is completely dependent on IL-2 (60, 61). To examine the effects of CTLA-4 cross-linking on these proteins, the induction of these regulatory elements was examined in CD4\(^+\) LNT cells 22 h after stimulation under various conditions. As previously reported with human T cells (56, 57, 64), CD3 engagement alone leads to cyclin D3, cdk4, and cdk6 protein expression, which are barely detectable in resting T cells (Fig. 5). CD28 cross-linking in the presence of anti-CD3 further up-regulates expression of these G\(_i\) kinases (Fig. 5). Interestingly, engagement of CTLA-4 dramatically inhibited the induction of these kinase components when cross-linked in conjunction with anti-CD3/CD28 and by CD3-ligation (Fig. 5). Degradation of the inhibitor p27 also occurs during the transition from G\(_i\) to S phase of the cell cycle upon T cell activation (60). p27 degradation initiated by anti-CD3 stimulation is inhibited by CTLA-4 cross-linking (Fig. 5). CTLA-4 engagement in conjunction with anti-CD3 plus anti-CD28, did not prevent the degradation of inhibitor p27 induced by anti-CD3 plus anti-CD28 (Fig. 5). Since p27 degradation is IL-2-dependent (60), this is most likely due to the remaining amounts of IL-2 produced in these cultures that leads to its degradation. Expression of p19, a p16 family member (55), was not affected by any of the stimulation conditions tested (Fig. 5).

FIGURE 4. Analysis of NF-AT and AP-1 binding activity of nuclear extracts from activated or nonactivated CD4\(^+\) T cells. EMSA of nuclear extracts of activated or nonactivated CD4\(^+\) T cells were incubated with the radioactive oligonucleotides containing the NF-AT (A), AP-1 (B), and GT-box-sites (C). The CD4\(^+\) T cells were incubated with a control Ab (1), or stimulated with anti-CD3 (2), anti-CD3/anti-CD28 (3), anti-CD3/anti-CD28/anti-CTLA-4 (4), or anti-CD3/anti-CTLA-4 (5). CsA was added (+) at 1 μg/ml. For each assay, nuclear extracts (4 μg) were incubated with the corresponding \(^32\text{P}\)-labeled probe containing the NF-AT site of the IL-2 promoter (A), or the AP-1-site (B). The data shown is representative of three experiments for NF-AT and two experiments for AP-1. \(^32\text{P}\)-labeled probe control is designated by “X”. The upper arrows indicate specific complexes; free probe is indicated.
Collectively, these results suggest that CTLA-4-mediated inhibition of T cell proliferation results, at least in part, from direct interference with induction of proteins that regulate cell cycle progression, rather than just by inhibiting the production of essential growth factor IL-2. To examine this possibility, we determined the effect of CTLA-4 engagement on proliferation of T cells in the presence of excess IL-2. As shown in Fig. 6, the addition of IL-2 (1–80 U/ml) increased proliferation under all culture conditions, reaching a plateau at ~10 U/ml IL-2. However, the addition of IL-2 (10–80 U/ml) did not reverse the inhibition of proliferation by CTLA-4. Indeed, despite the presence of excess IL-2, CTLA-4 engagement resulted in 50–80% inhibition of proliferation.

**Discussion**

Here, we have examined the mechanisms of CTLA-4-mediated inhibition of T cell activation. One issue that these experiments have addressed is the stage at which CTLA-4 can operate. Since CTLA-4 is not readily detectable in resting T cells, there has been a prevailing notion that CTLA-4 functions primarily to terminate ongoing T cell responses (34). Several previous reports have suggested that this might not be strictly correct, and that CTLA-4 might function quite early. First, as discussed previously, CTLA-4 mRNA and intracellular protein can be detected much earlier than surface expression. Second, it has been shown that CTLA-4 engagement by Ab can prevent several early manifestations of T cell activation, including expression of CD69, CD25, and IL-2 secretion (16, 17). Finally, it has been shown that CTLA-4 ligation can prevent accumulation of detectable amounts of IL-2 mRNA as early as 4 h after stimulation of resting human T cells by anti-CD3 and anti-CD28 (18). These findings all suggest that CTLA-4 might act very early to regulate T cell responses. Nevertheless, it remains possible that CTLA-4 can also play a role in regulating ongoing immune responses when CTLA-4 cell surface expression is maximal. These two possibilities are not mutually exclusive.

Our results clearly demonstrate that CTLA-4 inhibits the anti-CD3/CD28-stimulated accumulation of IL-2 mRNA by freshly isolated murine CD4+ T cells. Further, this inhibition is due to a block in IL-2 gene transcription and may be a consequence of inhibition of the activation and nuclear translocation of the transcription factor NF-AT. An important consequence of CD28-mediated costimulation is stabilization of mRNA of IL-2 and several other cytokines (38). Our results suggest that CTLA-4 ligation does not lead to IL-2 mRNA destabilization. This finding is consistent with the observation that CTLA-4 does not reverse CD28-mediated induction of Bcl-xL (18). These results strongly suggest that CTLA-4 does not merely counteract the costimulatory effects of CD28, but may act at different or additional points in T cell activation. The notion that CTLA-4 and CD28 costimulation may have independent effects is further supported by recent reports demonstrating that CTLA-4 can function in the absence of CD28 (21, 22).

Since CTLA-4 ligation can result in a very early inhibition of production of IL-2, this might suggest that the inhibition of T cell proliferation by CTLA-4 ligation might be attributed entirely to a lack of this essential growth factor (65). However, our observation that the addition of excess exogenous IL-2 failed to completely restore proliferation suggested that CTLA-4 might be inhibiting T cell proliferation independent of IL-2-mediated effects (Fig. 6, and Ref. 8). Indeed, we found that CTLA-4 ligation inhibits the production of components of the cell cycle machinery necessary for progression through the G0/G1 checkpoint. This result indicates that CTLA-4 can inhibit T cell proliferation at two stages, production of an essential growth factor and induction of critical components of the cell cycle machinery. It is of interest that CTLA-4 does not appear to interfere with the ability of scant amount of IL-2 produced upon cross-linking of CD3/CD28/CTLA-4 to support the degradation of the cell cycle inhibitor p27 (Fig. 5).

Our results were obtained by cross-linking CD3, CD28, and CTLA-4 using Ab-coated microspheres. Although the results clearly demonstrate an inhibitory role for CTLA-4, it will be important to examine the role of CTLA-4 in regulating T cell responses using physiological ligands. Recently, it has been reported...
that lymphocytic choriomeningitis virus-specific and 2C TCR transgene-expressing naive CD8+ T cells from CTLA-4−/−, and littermate control mice generated similar peptide-specific proliferative responses in vitro (66, 67). However, upon restimulation of the 2C TCR+ CD8+ T cells, the CTLA-4-deficient cells generated a dramatically greater proliferative response compared with the CTLA-4 wild-type T cells (67). In contrast, the proliferative response, IL-2 secretion, and the up-regulation of CD69 and CD25 by naive 2C TCR+ T cells stimulated by anti-CD3 and B7-transfected Chinese hamster ovary cells were inhibited by CTLA-4 cross-linking (7, 68). These seemingly disparate results suggest that the role of CTLA-4 in regulating T cell activation may depend on the availability of B7 ligands, strength of the TCR signal, and the activational history of the T cells.

Thus, CTLA-4 can exert its inhibitory effects at multiple points in T cell activation, including induction of transcription of an essential growth factor gene and interference with the production of critical components required for cell cycle progression. The mechanism appears to be more complex than mere reversal of CD28-mediated costimulation. The cytokine-dependent kinases erk4 and erk6 are induced by anti-CD3 alone, but can be further augmented upon CD28 costimulation (56, 62–64). CTLA-4 engagement can completely block anti-CD3-mediated induction. Finally, CTLA-4 ligation inhibits the induction of cyclin D3, which appears to be independent of CD28 costimulation.

It is clear that CD28 and CTLA-4 can influence the TCR/CD3-mediated signal. The molecular stage at which this occurs is unclear. There are several possibilities. It has been proposed that CD28 mediates a separate and parallel pathway that augments IL-2 transcription and T cell responses (69). Another possibility is that CD28 and TCR/CD3 signaling intersect distally at Jnk, thereby transcription and T cell responses (69). However, recently it has been shown that CD28/B7 signaling affects the CD3 signaling pathway directly (Ref. 71, and Y. Zhang and J.A.P., unpublished observations). Regardless of the mechanism, the results presented here support the notion that signals mediated by the TCR, CD28, and CTLA-4 are not discrete, but interact in a complex and dynamic way to influence the outcome of T cell stimulation.

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References


