Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) Interferes with Extracellular Signal-regulated Kinase (ERK) and Jun NH₂-terminal Kinase (JNK) Activation, but Does Not Affect Phosphorylation of T Cell Receptor ζ and ZAP70

By Concepcion Revilla Calvo, Derk Amsen, and Ada M. Kruisbeek

From the Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Summary

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an important regulator of T cell homeostasis. Ligation of this receptor leads to prominent downregulation of T cell proliferation, mainly as a consequence of interference with IL-2 production. We here report that CTLA-4 engagement strikingly selectively shuts off activation of downstream T cell receptor (TCR)/CD28 signaling events, i.e., activation of the microtubule-associated protein kinases (MAPKs) ERK and JNK. In sharp contrast, proximal TCR signaling events such as ZAP70 and TCR-ζ chain phosphorylation are not affected by CTLA-4 engagement on activated T cells. Since activation of the ERK and JNK kinases is required for stimulation of interleukin (IL)-2 transcription, these data provide a molecular explanation for the block in IL-2 production imposed by CTLA-4.

Activated T cells are subject to a number of downregulatory mechanisms designed to maintain T cell homeostasis, and several receptors involved in this process have been identified. Among these are the CD95 and the TNF receptor which both trigger apoptotic pathways (1, 2). Recently, it has become clear that CTLA-4 also downregulates T cell responses (for reviews see references 3, 4). Antibodies to CTLA-4 can, when crosslinked, prevent proliferation and IL-2 production of T cells stimulated through the TCR, whereas (blocking) Fab fragments of anti–CTLA-4 can actually enhance T cell responses (5–8). The physiological relevance of these findings is dramatically demonstrated by the phenotype of mutant mice lacking CTLA-4; such mice develop severe lymphoproliferative disease and massive lymphocytic infiltration and tissue destruction that is lethal by 3–4 wk of age (9, 10). The potential of CTLA-4 as a negative regulatory receptor is also illustrated by the recent findings that in vivo blockade of CTLA-4 retards the growth of an immunogenic tumor, implying augmented T cell–mediated antitumor immunity (11). Moreover, similar in vivo blockade of CTLA-4 has been found to markedly exacerbate disease in mice induced to develop experimental allergic encephalitis (EAE) (12).

CTLA-4 function is regulated by engagement with its ligands CD80 and CD86 on antigen-presenting cells (13). These molecules also regulate the function of CD28, a receptor promoting T cell activation and persistence of T cell responses by enhancing IL-2 production and expression of survival factors (for review see reference 4). How the same ligands can induce such opposite processes in T cells depending on the receptor engaged may be explained by the different expression patterns of CD28 and CTLA-4. Although CD28 is expressed constitutively on all T cells, CTLA-4 is expressed only after activation, reaching its peak after 48 h (6, 14–16). These expression patterns suggest that during the initial phase of T cell activation, CD28 may dominate the response to CD80/CD86. At later times after activation, CD80/CD86 molecules might downregulate the response by engaging CTLA-4. However, CTLA-4 can already function during the first 24 h of activation as demonstrated by antibody cross-linking studies, suggesting that CTLA-4 may also play a role in setting a threshold for activation (7).

Two nonmutually exclusive models have been proposed for the mode of action of CTLA-4 (17). First, CTLA-4 may specifically antagonize CD28 function, either by competing for CD80/CD86 molecules and/or by actively blocking CD28 signal transduction. The finding that the inhibitory effects of cross-linked anti–CTLA-4 can be overcome, to some extent, by addition of high doses of anti-CD28 might be interpreted as support for this model (6). Alternatively, CTLA-4 might interfere with TCR signaling as suggested by the hyperactivity of kinases associated with the TCR such as Lck and Fyn, as well as hyperphosphorylation.
The present study was designed to gain insight into the mechanism(s) used by CTLA-4 for negative regulation of T cell responses by directly examining signal transduction associated with CTLA-4 triggering. Using preactivated T cells, we find that CTLA-4 coengagement with the antigen receptor and CD28 leads to a reduction in the activities of both jun N H2-terminal kinase (JNK) and extracellular signal-regulated kinase 2 (ERK-2). Since ERK-2 activity induced by TCR engagement alone (i.e., in the absence of CD28 triggering) was also blocked by CTLA-4 engagement, these data demonstrate that CTLA-4 interferes with TCR signal triggering independently of any possible effects on CD28-mediated events. However, anti-CD3–induced transduction independently of any possible effects on data demonstrate that CTLA-4 interferes with TCR signal triggering alone (i.e., in the absence of CD28 binding) by TCR engagement alone (i.e., in the absence of CD28 triggering). As these kinases play crucial roles in induction of IL-2 transcription (19–21), this study was designed to gain insight into the mechanism(s) used by CTLA-4 for negative regulation of T cell responses by directly examining signal transduction associated with CTLA-4 triggering.

Materials and Methods

Mice. Lymph nodes were isolated from C57BL/6 mice (6–8-wk-old). The mice were bred at The Netherlands Cancer Institute (Amsterdam, The Netherlands) under specific pathogen-free conditions.

Media, Antibodies, and Other Reagents. Iscove’s modified Dulbecco’s medium ( Gibco BRL, Paisley, U.K.) was supplemented with 10% FCS (BioWhittaker, Inc., Verviers, Belgium), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 × 10−3 M 2-mercaptoethanol (Merck, Darmstadt, Germany).

The following antibodies were used in this study: 145C.11 (anti-CD3; reference 22) and M5/114 (anti-MHC II; Advanced Magnetics, Cambridge, MA); goat anti-CD28 (DAKO, Glostrup, Denmark); rabbit polyclonal antibodies (DAKO, Glostrup, Denmark); rabbit polyclonal IgG anti-TCR-ζ and ZAP70 in T cells from CTLA-4 knockout mice (18).

Production and purification of glutathione-S-transferase (GST)-ζ-PO-4 and 1E7.2 (anti-ZAP70) were provided by Drs. H. van Oers and A. Weiss.

Production and purification of glutathione-S-transferase (GST)-ζ-PO-4 and 1E7.2 (anti-ZAP70) were provided by Drs. H. van Oers and A. Weiss.

Cell Stimulation. T cells were purified from lymph node cell suspensions as follows. Nylon wool passed (NWP) lymph node cells were incubated with anti-class II mAb (M5/114). The NWP lymph node cells were depleted of antibody binding cells through magnetic bead depletion using goat anti-mouse Ig beads (Advanced Magnetics, Cambridge, MA) and sheep anti-rat Ig beads (Dynal, Oslo, Norway). T cell preparations were typically >98% pure by anti-CD3 staining.

To induce CTLA-4 expression, purified T cells (4 × 106 cells/well) were preactivated in 24-well plates coated with anti-CD3 and anti-CD28 mAbs (10 μg/ml each). After 40 h, cells were harvested and then rest for 5 h in 10% FCS-containing medium at 37°C. Purified naive (fresh cells rested in vitro for 5 h in 10% FCS-containing medium at 37°C) or preactivated T cells were incubated in medium with anti-CD3, anti-CD28, and anti-CTLA-4 (each at 10 μg/ml) at the indicated combinations for 30 min on ice. Cells were washed twice with cold medium and goat anti-hamster antibody was added at 10 μg/ml in warm (37°C) medium. After this, cells were incubated for the indicated times at 37°C. The reactions were stopped by addition of ice-cold PBS after which the cells were spun down. Subsequently, cells were lysed as described below.

ERK-2 and JNK Kinase Assays. Cells were lysed for 30 min on ice with whole cell extract lysis buffer (25 mM HEPES at pH 7.7, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM diethiothreitol (DTT), 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 2 μg/ml of leupeptin, and 1 mM of PM SF (25). Lysates were cleared by centrifugation at 14,000 g for 10 min at 4°C. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce), and 10 μg of total protein/lane was used for immunoprecipitation. Whole cell extracts were diluted to get as final concentration of the buffer: 20 mM HEPES at pH 7.7, 75 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 2 μg/ml of leupeptin, and 1 mM of PM SF. For JNK precipitation, cells were incubated overnight (0/8) at 4°C with 10 μg of GST–c-Jun bound to glutathione-agarose beads (Sigma Chemical Co.). Precipitates were washed 4 times with HEPES binding buffer (20 mM HEPES at pH 7.7, 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, and 0.05% Triton X-100), and subsequently resuspended in 30 μl of kinase buffer (20 mM HEPES at pH 7.6, 20 mM MγC12, 20 mM β-glycerophosphate, 20 mM para-nitrophenyl phosphate, 0.1 mM Na3VO4, and 1 mM of PM SF) containing 20 μM cold ATP and 5 μCi of γ[32P]ATP. After 20 min at 37°C the reaction was stopped by washing the beads 3 times with HEPES binding buffer. GST–c-Jun was eluted and resolved on 10% SDS-PAGE gel.

For ERK-2 immunoprecipitation, the cells were incubated o/n at 4°C with 1 μg anti-ERK-2 bound to protein A–Sepharose beads (Pharmacia Biotech, Upsala, Sweden). Immunoprecipitates were washed 3 times with 50 mM TRIS, pH 7.5, 100 mM NaCl, 1 μM EGTA, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, and once with 20 mM HEPES of pH 7.5, 0.5 mM MγC12, 10 mM MγC12, 1 μM EGTA, 20 mM β-glycerophosphate, and 0.1 mM Na3VO4. Kinase reactions were performed at 37°C in 30 μl of kinase buffer containing 20 μM HEPES of pH 7.5, 0.5 mM NaCl, 10 mM MγC12, 1 μM EGTA, 1 mM DTT, 0.3 mg/ml MBP, 25 μM cold ATP, and 5 μCi of γ[32P]ATP. After 20 min, the reaction was finished by adding 5 μl of 5× sample buffer. Samples were boiled and resolved on 12.5% SDS-PAGE. Phosphorylation of GST–c-Jun and MBP was visualized and the radioactivity of each band quantified using a phosphorimager.

For ERK-2 and JNK immunoblots, proteins from total lysates were resolved on 10% SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking, membranes were blotted with monoclonal rabbit anti-ERK-2 or anti-JNK antibodies, respectively, followed by a swine anti-rabbit peroxidase-conjugated antibody. Blots were developed using enhanced chemiluminescence (Amersham Int., Buckinghamshire, U.K.). TCR-ζ Chain and ZAP70 Immunoprecipitation and Immunoblotting. Preactivated T cells were lysed in lysis buffer containing 20
mM Tris-HCl of pH 7.6, 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors. After 30 min of incubation on ice, lysates were cleared by centrifugation and incubated 0/n at 4°C with anti-TCR-ζ chain or anti-ZAP70 antibodies bound to Sepharose-protein A beads. Beads were washed 3 times with lysis buffer and proteins eluted by boiling in 2× sample buffer. Proteins were resolved in SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts), and blocked 0/n in PBS, 0.2% Tween 20, and 1% BSA. Tyrosine phosphorylation was detected with antiphosphotyrosine antibody followed by rabbit anti-mouse peroxidase-conjugated secondary antibody. Blots were developed using enhanced chemiluminescence.

Results

ERK2 Activity Is Reduced upon CTLA-4 Engagement. Studying the individual contributions to T cell activation of the CD28 and CTLA-4 receptors is complicated by the fact that both receptors are triggered by the same ligands, the CD80/CD86 molecules. However, the availability of mAbs to CD28 and CTLA-4 has made it possible to separately investigate the functions of these receptors. The inhibitory role of CTLA-4 in T cell activation has now been visualized in several studies (5–8). The block imposed by CTLA-4 engagement on T cell activation predominantly results from interference with IL-2 production (6, 8), resulting in inhibition of cell cycle progression.

The ERK family of MAP kinases has been found to be required for stimulation of IL-2 transcription (19, 26). Therefore, we investigated whether the activity of ERK2 would be affected by CTLA-4 triggering. Because CTLA-4 has been reported to be expressed only after activation, peaking after 2 d (6), we first preactivated T cells purified from lymph nodes for 40 h on plates coated with anti-CD3 and anti-CD28. After harvesting, we incubated the preactivated cells in fresh medium for an additional 5 h, as we observed that immediately after the 40-h activation period, ERK2 and JNK were too strongly activated to allow visualization of induction by TCR triggering (data not shown). After this 5-h incubation, cells were stimulated with different combinations of antibodies as indicated, and lysed. In vitro kinase reactions were performed with anti-ERK2 immunoprecipitates from these lysates. As has been shown previously (20, 27), efficient ERK2 activation can be achieved by triggering the TCR alone (Fig. 1A). However, in lysates derived from cells on which CTLA-4 is coengaged together with the antigen receptor, ERK2 activity is almost abrogated. Shown here are the results obtained with cells lysed 1 min after cross-linking the antibodies. In most experiments at this time point, anti-CD3-induced ERK2 activity has reached its maximum, being undetectable by 5 min after stimulation. However, in experiments in which anti-CD3-induced ERK2 activity persisted longer, this activity was still reduced by CTLA-4 engagement at 5 min after triggering (data not shown). The pronounced anti-CTLA-4-induced reduction in ERK2 activity in preactivated cells is contrasted with results in freshly isolated T cells in which no effect of addition of anti-CTLA-4 on ERK2 activity was observed (Fig. 1B). These findings are consistent with the reported requirement for activation of CTLA-4 expression.

In contrast to earlier published results that suggest that ERK2 activity is not affected by CD28 triggering (20, 27), we consistently found that in cells stimulated with anti-CD3 and anti-CD28, ERK2 activity is somewhat higher (Fig. 1). Moreover, we found that ERK2 activity induced in the presence of anti-CD28 usually persisted longer than ERK2 activity induced by TCR triggering alone, reaching its peak by 10 min after activation (Fig. 1B). Interestingly, an effect of CD28 signaling on ERK2 activity is also revealed by the finding that 1 min after stimulation the inhibition of ERK2 activity/activation by CTLA-4 is prevented by anti-CD28 (compare Figs. 1A and 2). At later time points, however, anti-CD3- and anti-CD28-induced ERK2 activity is also downregulated by CTLA-4 (Fig. 2) with almost complete inhibition at 10 min. Together, these results demonstrate that CTLA-4 on preactivated T cells downregulates TCR-induced ERK2 activity, either by interfering with activation of this kinase or by directly modulating ERK2 kinase activity.

JNK Activity Is Reduced upon CTLA-4 Engagement. Like the ERKs, the JNK family of MAP kinases have been implicated in induction of IL-2 gene transcription, as these enzymes phosphorylate the NH₂ terminus of c-Jun and thereby enhance its transcriptional activity (20, 21, 25, 28, 29). We therefore tested whether the activity of JNK might also be regulated by CTLA-4. JNKs bind with high affinity to c-Jun, such that these kinases can be precipitated from cell lysates with immobilized GST–c-Jun coupled to beads (25), after which in vitro kinase reactions can be performed. It has been shown in both Jurkat T cells and in T
CTL8-A4 is an important regulator of peripheral T cell homeostasis by virtue of its ability to negatively regulate T cell expansion. Although the functional consequences of CTL8-A4 engagement have been studied extensively in

CTLA-4 Does Not Affect Early Parameters of TCR Triggering. The data presented above demonstrate that CTLA-4 imposes a block in signal transduction upstream or at the level of ERK2 and JNK. Furthermore, these findings document that CTLA-4 has an effect on signal transduction events induced by TCR triggering independent of CD28 engagement, as ERK2 activity induced by anti-CD3 alone was reduced upon coengagement of CTLA-4 with CD3 (Fig. 1A). A role for CTLA-4 in countering early activation events associated with TCR signal transduction is also suggested by findings in T cells derived from CTLA-4-deficient mice. Thus, in CTLA-4−/− cells, proteins such as TCR-ζ and ZAP70 are constitutively hyperphosphorylated (18). For these reasons, we directly investigated whether anti-CD3-induced TCR-ζ phosphorylation and ZAP70 phosphorylation are affected by CTLA-4 triggering.

The TCR-ζ chain contains three so-called ITAMs (immunoreceptor tyrosine-based activation motifs) sequences that are necessary for coupling the TCR to the intracellular signaling machinery (30–32). Upon TCR activation, these motifs become phosphorylated on tyrosine residues as a consequence of which the electrophoretic mobility of the protein changes (33, 34). We immunoprecipitated TCR-ζ from lysates obtained as described above using an antibody specific for phosphorylated TCR-ζ. After immunoblotting the precipitates with an antibody against phosphotyrosine, bands of 21 kD and p23 kD were visualized, representing different phosphorylated isoforms of TCR-ζ (Fig. 4A; reference 34). Although the 21-kD band was also present in unstimulated cells as previously reported for fresh lymph node T cells (35, 36), the p23 band specifically appeared after CD3 triggering. Importantly, at none of the time points examined was the intensity of either one of these two phospho-ζ bands altered upon coengagement of CTLA-4 with CD3 (Fig. 4A). Yet, in these same cells, ERK2 activity was again abrogated by CTLA-4 engagement (data not shown). We conclude that ζ phosphorylation is not affected by CTLA-4.

Phosphorylation of TCR-ζ allows binding of ZAP70 via its SH2 domains (37). Moreover, the recruited ZAP70 molecules become phosphorylated on tyrosine residues shortly after TCR triggering, leading to activation of this kinase (36, 38, 39). To investigate potential effects of CTLA-4 on ZAP70 phosphorylation, we immunoprecipitated this kinase and immunoblotted with an antiphosphorylated antibody. As shown in Fig. 4B, phosphorylation of ZAP70 at 1 min after stimulation was not affected by CTLA-4 engagement. Similarly, we found no changes in the phosphorylation state of this kinase at later time points (data not shown). Together, these data strongly suggest that the block imposed by CTLA-4 resulting in decreased ERK and JNK activity lies downstream of the TCR proximal events that result in phosphorylation of TCR-ζ and ZAP70.

Discussion

CTLA-4 is an important regulator of peripheral T cell homeostasis by virtue of its ability to negatively regulate T cell expansion. Although the functional consequences of CTLA-4 engagement have been studied extensively in

Figure 2. Combined triggering of CD3 and CD28 delays downregulation of ERK2 activity by CTLA-4. Preactivated T cells were coated with different combinations of antibodies as in Fig. 1 and lysed 1, 5, or 10 min after addition of warm cross-linking antibody. ERK2 was immunoprecipitated and in vitro kinase reactions were performed (top). The same lysates were tested for ERK2 protein abundance (middle). ERK2 activities were quantified using a phosphoimager and represented as relative values compared to ERK2 activities from unstimulated cells (unstimulated = 1; bottom).

Figure 3. CTLA-4 reduces JNK activity induced by CD3 and CD28 triggering. Preactivated T cells were coated with different combinations of antibodies and lysed 10 min after addition of warm cross-linking antibody. Lysates were tested for JNK activity by precipitation with GST-c-jun precoupled to glutathione beads followed by in vitro kinase reactions as described in Materials and Methods. Phosphorylated GST-c-jun is indicated by the arrow (top). The same lysates were tested for JNK protein abundance on immunoblot (middle). JNK activities were quantified using a phosphoimager and represented as relative values compared to JNK activity from unstimulated cells (unstimulated = 1; bottom). Data for the 10-min time point are shown because no JNK activity could be measured at earlier times.
of CD28 signaling (17). This proposal resulted from the May function might be by counteracting the consequences of defective ERK and JNK activation, and occurs downstream of immediate TCR signaling events.

We found that antigen receptor and CD28-mediated transduction events associated with T cell activation. We directly investigate how CTLA-4 engagement influences signal transduction resulting from ligation of this receptor. Some investigators have reported a receptor-ligation-inducible association of PI3 kinase with the cytoplasmic tail of CTLA-4 (40). However, as the same kinase also binds to the cytoplasmic tail of CD28 (41), it seems unlikely that association of this kinase with CTLA-4 would explain its inhibitory effects on induction of T cell proliferation. It has also been suggested that the role of PI3 kinase binding to CTLA-4 may be to regulate transport of this receptor, most of which is localized in intracellular vesicles, to the cell surface (42–44). Other investigators have found that a phosphatase termed Syp (also known as SHP2 or PTP1D) can be communoprecipitated with CTLA-4 (18). Furthermore, it was shown that these precipitates could dephosphorylate the adapter protein Shc (see below) in vitro. In apparent agreement with a role for a phosphatase in the function of CTLA-4, these same investigators showed that in T cells derived from CTLA-4-deficient mice, the kinases Fyn, Lck, and ZAP70 were hyperactivated (18). Also, other proteins such as TCR-ζ and Shc, were constitutively hyperphosphorylated in CTLA-4-/- mice (18). However, rather than being a direct consequence of the absence of CTLA-4, such observations might also result from the hyperactivated state of T cells in CTLA-4-deficient mice (9, 10).

In the present study, we used preactivated T cells to directly investigate how CTLA-4 engagement influences signal transduction events associated with T cell activation. We found that antigen receptor and CD28-mediated activation of the MAP kinases ERK2 and JNK is impeded upon coengagement of CTLA-4. In sharp contrast, phosphorylation of TCR-ζ and ZAP70 were unaffected by CTLA-4 triggering. CTLA-4-induced T cell inactivation is thus linked to defective ERK and JNK activation, and occurs downstream of immediate TCR signaling events.

It has been suggested that one way in which CTLA-4 may function might be by counteracting the consequences of CD28 signaling (17). This proposal resulted from the observation that high doses of an antibody to CD28 can, at least to some extent, rescue T cell proliferation from anti-CTLA-4-induced inhibition (6). The present data, however, unequivocally demonstrate that CTLA-4 interferes with signal transduction events induced by the TCR independent of CD28 triggering. These findings are therefore consistent with the hypothesis that CTLA-4 interferes directly with downstream TCR signaling events. In addition, CTLA-4 may affect signal transduction through CD28, as suggested by the reduction in JNK activity induced by anti-CTLA-4. Whether this reflects a direct or indirect effect remains to be investigated. The “rescuing” effects of anti-CD28 on T cell proliferation may in fact be the result of modulation of TCR signaling by CD28. In support of this possibility, we found anti-CD28 to affect both the strength and the persistence of ERK2 activity induced by TCR signaling. Indeed, it also prevented the blockade of TCR-induced ERK2 activity by CTLA-4 at early time points after activation. Although at later time points the CTLA-4-induced block in ERK2 activity did manifest itself despite CD28 engagement, it is conceivable that at lower concentrations of anti-CTLA-4 or higher concentrations of anti-CD28, rescuing effects from CD28 became dominant. A dominance of CD28 over CTLA-4 might be relevant in situations in which CTLA-4 expression on T cells is still low and CD80/CD86 expression is relatively high.

In view of a recent suggestion (45) that CTLA-4 can actually also provide positive costimulatory signals in T cell activation, it will be interesting to compare the signaling pathways emanating from TCR and CTLA-4 under conditions that visualize either positive or negative effects of CTLA-4 engagement. Our experiments were performed under conditions in which CTLA-4 engagement results in an abrogation of IL-2 production, and such findings have been documented by many laboratories (for review see references 3, 17). A role for CTLA-4 in blocking IL-2 production is also consistent with the phenotype of CTLA-4-/- mice (9, 10). Nevertheless, a positive regulatory effect of CTLA-4 may,
under other conditions (45), dominate the response, although the latter studies (45) did not exclude an adhesive interaction between CTLA-4 and CD80, allowing other costimulatory receptors to exert their effect.

Another issue that remains to be resolved is to which extent the antibody-mediated cross-linking of TCR/CD28/CTLA-4 used in this study truly reflect physiological, ligand-triggered events. An example of the different outcome between antibody- and ligand-mediated triggering was revealed some years ago by the Cantrell laboratory (ICRF, London, UK); although antibody-mediated triggering of CD28 resulted in p21ras activation, CD80-mediated triggering did not (46). An alternative explanation for those observations, however, might be that the CD80 ligand triggered both CD28 and CTLA-4, and actively abrogated p21ras activation. Antibodies to CD28, on the other hand, would allow activation of p21ras to be revealed. Until reciprocal mutants of CD80 or CD86 become available that selectively bind to either CD28 or CTLA-4, the issue of whether the anti-CTLA-4 triggered inhibitory effects on downstream TCR signaling events mimic ligand-induced events remains to be tested. It should be kept in mind, however, that the observed modulatory effect of anti-CTLA-4 on the MAPKs is consistent with the results of earlier functional studies (5–12) both in vitro and in vivo.

The inhibition of T cell proliferation by CTLA-4 engagement can be overcome by addition of IL-2 (8; data not shown). This suggests that the blockade of proliferation by CTLA-4 is predominantly a consequence of its interference with IL-2 production. The data presented here provide a molecular explanation for this interference, since both the ERKs and the JNKs are involved in induction of IL-2 gene transcription. A well documented role for the ERKs is phosphorylation of ternary complex factor (TCF)/Etk-1 (47–49), stimulating it to activate fos transcription. JNKs, on the other hand, enhance the transcriptional activity of c-Jun by NH2-terminal phosphorylation (25). Together, Fos and Jun proteins form AP1 complexes that bind to a functionally important AP1 site in the IL-2 promoter and, in addition, participate in formation of NFAT and NF-IL-2 (50–53). Transfection studies have established that, at least in transformed T cell lines, ERK activity is indeed required for IL-2 promoter-driven gene transcription (19). Furthermore, JNK activity also appears to be required, since transfected Jun mutants that cannot be phosphorylated inhibit IL-2 promoter activity in luciferase reporter assays (20).

Although in theory inhibition of either pathway alone might be sufficient to severely restrict IL-2 production, inhibition of both pathways probably results in a more pronounced block. The simplest explanation for how both of these kinases are inhibited would be that CTLA-4 inhibits a target that is commonly involved in turning on both cascades. As TCR triggering is required for activation of ERKs as well as of JNKs (20, 27, and this paper), this target would have to be activated by TCR triggering alone and lie downstream of TCR-ζ phosphorylation. In this respect, it would be attractive to speculate that CTLA-4 inhibits TCR signal transduction at the level of or upstream of p21ras. It is well established that in many cell types, including T cells, p21ras activates and is required for activation of ERK1 and ERK2 (54–56). Moreover, although Ras as activity alone is insufficient to activate JNK in T cells, it has been reported that overexpression of a gene encoding dominant negative Ras does inhibit JNK activation (21). Consistent with an effect on Ras, others have observed that anti-Syp as well as anti-CTLA-4 immunoprecipitates could, in vitro, dephosphorylate the adaptor protein Shc (18), a protein that is used by various receptor tyrosine kinases for activation of p21ras via its association with Grb2-Sos complexes (57–60). Although the in vivo relevance of this in vitro dephosphorylation activity still remains to be established, these data might provide an explanation for the results presented here by us. However, there is some controversy about whether Shc is in fact involved in TCR-mediated Ras activation. Thus, although T cell hybridomas complexes of Shc-Grb2-SOS complexes were found after TCR cross-linking (61), such complexes could not be detected in TCR-activated peripheral blood T cells (62). Instead, in these latter cells, Grb2 and Sos, were found complexed with another adapter of ~36–38 kDa, possibly the recently cloned p36 (63, 64). Furthermore, additional mechanisms, not involving Grb2-Sos, exist for TCR-mediated Ras activation in T cells, including inhibition of Ras as GTP-activating proteins (Ras-GAP) as well as PKC-mediated regulation (65, 66). Finally, in signaling from several receptor tyrosine kinases, the phosphatase Syp has actually been reported to stimulate rather than inhibit growth factor-induced ras and MAPK activation (67, 68). Thus, it is unclear whether possible effects from CTLA-4-associated Syp would provide a satisfactory explanation for the inhibition of ERK and JNK activation we here report. Nevertheless, involvement of a phosphatase seems conceptually appealing, given the role of phosphatases in inhibitory signaling by NK receptors and FcγRIIB (69).

Modulation of CTLA-4 activity promises to be a powerful option for manipulation of immune responses. The possibilities for this modulation are currently confined to the use of anti-CTLA-4 antibodies. Therefore, a more detailed understanding is required of the mechanisms used by this receptor for its downregulation of IL-2 production. Possibly a molecular understanding of CTLA-4 action may offer novel opportunities for downregulating undesired responses or stimulating useful ones. In the present study, we have shown that the molecular target(s) for CTLA-4-mediated interference with IL-2 production lie(s) upstream of ERK and JNK, but downstream of TCR-ζ. Identification of this target will be a major focus for the future.

We gratefully acknowledge Dr. N.S.C. van Oers and Dr. A. Weiss for providing anti-TCR-ζ and ZAP70 antibodies. Furthermore, we thank Dr. W. Li for providing technical advice on JNK and ERK kinase assays.
References

1. Abbas, A.K. 1996. Die and let live: eliminating dangerous lymphocytes. Cell. 84:655–658.

2. Zheng, L., G. Fisher, R.E. Miller, J. Peschon, D.H. Lynch, and M.J. Lenardo. 1995. Induction of apoptosis in mature T cells by tumour necrosis. Nature (Lond.). 377:348–352.

3. Tivol, E.A., A.N. Schweitzer, and A.H. Sharpe. 1996. Co-stimulation and autoimmunity. Curr. Opin. Immunol. 8:822–830.

4. Lenschow, D.J., T.L. Walunas, and J.A. Bluestone. 1996. CD28/B7 system of T cell costimulation. Annu. Rev. Immunol. 14:233–258.

5. Walunas, T.L., D.J. Lenschow, C.Y. Bakker, P.S. Linsley, G.J. Freeman, J.M. Green, C.B. Thompson, and J.A. Bluestone. 1996. CTLA-4 can function as a negative regulator of T cell activation. Immunity. 1:405–413.

6. Krummel, M.F., and J.P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J. Exp. Med. 182:459–465.

7. Krummel, M.F., and J.P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J. Exp. Med. 183:2533–2540.

8. Walunas, T.L., C.Y. Bakker, and J.A. Bluestone. 1996. CTLA-4 ligation blocks CD28-dependent T cell activation. J. Exp. Med. 183:2541–2550.

9. Tivol, E.A., F. Borriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone, and A.H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 3:541–547.

10. Waterhouse, P., J.M. Penninger, E. Timms, A. Wakeham, A. Shahnian, K.P. Lee, C.B. Thompson, H. Riesser, and T.W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. Science (Wash. DC). 270:985–988.

11. Leach, D.R., M.F. Krummel, and J.P. Allison. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. Science (Wash. DC). 271:1734–1736.

12. Perrin, P.J., H. Maldonado, T.A. Davis, C.H. June, and M.K. Racker. 1996. CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. J. Immunol. 157:1333–1336.

13. Linsley, P.S., J.L. Greene, W. Brady, J. Bayorath, J.A. Ledbetter, and R. Peach. 1994. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. Immunol. 1:793–801.

14. Linsley, P.S., J.L. Greene, P. Tan, J. Bradshaw, J.A. Ledbetter, C. Aisetti, and N.K. Damle. 1996. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J. Exp. Med. 176:1595–1604.

15. Lindsten, T., K.P. Lee, E.S. Harris, B. Petryniak, N. Craighead, P.J. Reynolds, D.B. Lombard, G.J. Freeman, L.M. Nailer, G.S. Gray, C.B. Thompson, and C.H. June. 1993. Characterization of CTLA-4 structure and expression on human T cells. J. Immunol. 150:3489–3499.

16. Perkins, D., Z. Wang, C. Donovan, H. He, D. Mark, G. Guan, Y. Want, T. Walunas, J. Bluestone, J. Listman, and P.W. Finn. 1996. Regulation of CTLA-4 expression during T cell activation. J. Immunol. 155:4154–4159.

17. Bluestone, J.A. 1997. Is CTLA-4 a master switch for peripheral T cell tolerance? J. Immunol. 158:1989–1993.

18. Marenghi, L.E.M., P. Waterhouse, G.S. Duncan, H.-W. Mittmucker, G.-S. Feng, and T.W. Mak. 1996. Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. Science (Wash. DC). 272:1170–1174.

19. Whitehurst, C.E., and T.D. Geppert. 1996. MEK1 and the extracellular signal-regulated kinases are required for the stimulation of IL-2 gene transcription in T cells. J. Immunol. 156:1020–1029.

20. Su, B., E. Jacinto, M. Hibi, T. Kalunski, M. Karin, and Y. Ben-Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. Cell. 77:727–736.

21. Su, B., and M. Karin. 1996. Mitogen-activated protein kinase cascades and regulation of gene expression. Curr. Opin. Immunol. 8:402–411.

22. Leo, O., M. Foo, D.H. Sachs, and L.E. Samelson. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. Proc. Natl. Acad. Sci. USA. 84:1374–1378.

23. Gross, J.A., E. Calles, and J.P. Allison. 1992. Identification and distribution of the costimulatory receptor CD28 in the mouse. J. Immunol. 149:380–382.

24. Smith, D.B., and K.S. Johnson. 1988. Single step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene (Amst.). 67:31–40.

25. Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-jun activation domain. Genes Dev. 7:2135–2148.

26. O’waki, H., R. Varma, B. Gillis, J.T. Bruder, U.R. Rapp, L.S. Davis, and T.D. Geppert. 1993. Raf-1 is required for T cell IL-2 production. EMBO (Eur. Mol. Biol. Organ.) J. 12:4367–4373.

27. Li, W., C.D. Wahley, A. Mondino, and D.L. Mueller. 1996. Characterization of antitumor immunity by CTLA-4 blockade. Proc. Natl. Acad. Sci. USA. 84:655–658.

28. Derijard, B., M. Hibi, I.H. Wu, T. Barbet, B. Su, T. Deng, M. Karin, and R.J. Davis. 1994. JNK1, a protein ki-
nae stimulated by UV light and Ha-R as that binds and phospho-
rylates the c-Jun activation domain. C.ell. 76:1025–1027.
29. Kyriakis, J.M., P. Banerjee, E. N. Ikolaikake, T. Dai, E.A. Rube-
bie, M.F. Ahmad, J. A. Vruch, and J. R. W. Oodgett. 1994. The stress-
activated protein kinase subfamily of c-Jun kinases. Nature (Lon-d.). 369:156–160.
30. Eth, M. 1989. Antigen receptor tail clue. Nature (Lon-d.). 338:383–384.
31. Weiss, A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein–tyrosine kinases. Cell. 73: 209–212.
32. Borst, J., G.S. Brouns, E. de Vries, M.C.M. Verschuren, D.Y. Mazon, and J.J.M. van Dongen. 1996. Composition and function of T-cell receptor and B-cell receptor complex-
exes on precursor lymphocytes. Immunol. Rev. 8:181–190.
33. Koyasu, S., D. J. Cunonkey, L.K. Clayton, S. Abraham, B. Yandava, T. Kakagiri, P. Moingeon, P. Yamamoto, and L.E. R Reinherz. 1992. Phosphorylation of multiple CD3ζ tyrosine residues leads to formation of pp21 in vitro and in vivo. J. Mol. Biol. 267:3375–3381.
34. Qian, D., I. Griswold-Prenner, M.R. Rosner, and F.W. Fitch. 1993. Multiple components of the T cell antigen re-
ceptor complex become tyrosine-phosphorylated upon activa-
tion. J. Biol. Chem. 268:4486–4493.
35. Van Oers, N.S.C., W. Tao, J.D. Watts, P. Johnson, R. Ae-
bersold, and H.-S. Teh. 1993. Constitutive tyrosine phos-
phorylation of the T-cell receptor (TCR)ζ subunit: regulation of TCR-associated protein tyrosine kinase activity by TCRζ. Mol. Cell. Biol. 13:5771–5780.
36. Van Oers, N.S.C., N. Killeen, and A. Weiss. 1994. ZAP-70 is con-
stitutively associated with tyrosine-phosphorylated TCRζ in murine thymocytes and lymph node T cells. Immunity. 1: 675–685.
37. Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: A 70 kD protein–tyrosine kinase that associates with the TCRζ chain. Cell. 71:649–662.
38. Chan, A.C., M. Dalton, R. Johnson, G.-H. Kong, T. Wang, R. Thoma, and T. Kuromoski. 1995. Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. EMBO (Eur. Mol. Biol. Org.) J. 14:2499–2508.
39. Wange, R.L., R. Guitian, N. Isakov, J.D. Watts, R. Aeber-
sold, and L.E. Samelson. 1995. Activating and inhibitory muta-
tions in adjacent tyrosines in the kinase domain of ZAP-70. J. Biol. Chem. 270:18730–18733.
40. Schneider, H., K.V.S. Prasad, S.E. Shoelson, and C.E. Rudd. 1995. CTLA-4 binding to the lipid kinase phosphatidylinosito-
tol 3-kinase in T cells. J. Exp. Med. 181:351–356.
41. Rudd, C.E. 1996. Upstream-downstream: CD28 cosignaling pathways and T cell function. Immunity. 4:527–634.
42. Leung, H.T., J. Bradshaw, J.S. Cleaveland, and P.S. Linsley. 1995. Cytotoxic T lymphocyte–associated molecule-4, a high-
avidity receptor for CD80 and CD86, contains an intracellu-
ar localization motif in its cytoplasmic tail. J. Biol. Chem. 270:25107–25114.
43. Linsley, P.S., J. Bradshaw, J. Greene, R. Peach, K.L. Bennett, and R.S. Miltner. 1996. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. Immunity. 4:535–543.
44. Hutchcroft, J.E., and B.E. Bierer. 1996. Signaling through CD28/CTLA-2 family receptors. Puzzling participation of phosphatidylinositol-3 kinase. J. Immunol. 156:4071–4074.
45. Wu, Y., Y. Guo, A. Huang, P. Zheng, and Y. Liu. 1997. CTLA-4-B7 interaction is sufficient to costimulate T cell clonal expansion. J. Exp. Med. 185:1327–1335.
46. Nunez, J.A., Y. Collette, A. Truneh, D. Olive, and D.A. Cantrell. 1994. The role of p21MAPK in CD28 signal transduc-
tion: triggering of CD28 with antibodies, but not the ligand B7-1, activates p21MAPK. J. Exp. Med. 180:1067–1076.
47. Marais, R., J. Wynne, and R. Teisram. 1993. The SH2 domain of the accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cél. 73:381–393.
48. Hipskind, R.A., D. Büscher, A. Nordhein, and M. Baccar-
ini. 1994. Ras MAP kinase-dependent and -independent signal-
ign pathways target distinct ternary complex factors. Genes Dev. 8:1803–1816.
49. Hill, C.S., and R. Teisram. 1995. Transcriptional regulation by extracellular signals mechanisms and specificity. Cél. 80: 199–211.
50. Crabtree, G.R. 1989. Contingent genetic regulatory events in T lymphocyte activation. Scien. (Wash. DC). 243:355–361.
51. Jain, J., P.G. McCaffrey, V.E. Valge-Archer, and A. Rao. 1992. Nuclear factor of activated T cells contains Fos and Jun. Nature (Lon-d.). 356:801–804.
52. Uliman, K.S., J.P. Northrup, A. Adam, and G.R. Crabtree. 1993. Jun family members are controlled by a calcium-regu-
lated, cyclosporin A–sensitive signaling pathway in activated T lymphocytes. Genes Dev. 7:186–196.
53. Northrop, J.P., K.S. Uliman, and G.R. Crabtree. 1993. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. J. Biol. Chem. 268:2917–2923.
54. Rayter, S.I., M. Wodrow, S.C. Lucas, D.A. Cantrell, and J. Downward. 1992. P21跟他 mediates control of IL-2 gene pro-
moter function in T cell activation. EMBO (Eur. Mol. Biol. Org.) J. 11:4549–4556.
55. Izquierdo, M., S.J. Leeners, C.J. Marshall, and D. Cantrell. 1993. p21跟他 couples the T cell antigen receptor to extracellu-
lar signal-regulated kinase 2 in T lymphocytes. J. Exp. Med. 178:1199–1208.
56. Baldari, C.T., A. Heguy, and J.L. Telford. 1993. Ras as protein activity is essential for T-cell antigen receptor signal transduc-
tion. J. Biol. Chem. 268:2693–2695.
57. Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, and J. Downward. 1993. Epidermal growth factor exchange protein with Grb2 is implicated in tyrosine kinase signaling. Cell. 73:611–620.
58. Buday, L., and J. Downward. 1993. Epidermal growth factor regulates p21跟他 through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cél. 73:611–620.
59. Egan, S.E., B.W. Giddings, M.W. Brooks, L. Buday, A.M. Sizeland, and R.A. Weinberg. 1993. Association of Sos R as exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature (Lon-d.). 360:689–692.
60. Li, N., A. Batzer, R. Daly, Y. Yajnik, E. Skolnik, P. Char-
din, D. Bar-Sagi, B. Margolis, and J. Schlessinger. 1993. Gua-
mine-nucleotide-releasing factor hSolS binds to Grb2 and links receptor tyrosine kinases to ras signaling. Nature (Lon-d.). 363: 85–88.
61. Ravichandran, K.S., K.K. Lee, Z. Songyang, L.C. Cantley, P. Burn, and S.J. Burakoff. 1993. Interaction of Shc with the zeta chain of the T cell receptor upon T cell activation. Scien. (Wash. DC). 262:902–905.
62. Osman, N., S. Lucas, C.H. Turner, and D. Cantrell. 1995. A comparison of the interaction of Shc and tyrosine kinase ZAP-70 with the T cell antigen receptor ζ chain tyrosine-based activation motif. J. Biol. Chem. 270:13981–13986.

63. Buday, L., S.E. Egan, P.R. Viciana, D.A. Cantrell, and J. Downward. 1994. A complex of Grb2 adaptor protein, Sox exchange factor and a 36 kDa membrane bound tyrosine phosphoprotein is implicated in Ras activation in T cells. J. Biol. Chem. 269:9019–9023.

64. Huang, X., Y. Li, K. Tanaka, K.G. Moore, and J.I. Hayashi. 1995. Cloning and characterization of Lnk, a signal transduction protein that links T-cell receptor activation signal to phospholipase C-γ1, Grb2, and phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. USA. 92:11618–11622.

65. Izquierdo, M., J. Downward, J.D. Graves, and D.A. Cantrell. 1992. Role of protein kinase C in T-cell antigen receptor regulation of p21ras: evidence that two p21ras regulatory pathways coexist in T cells. Mol. Cell. Biol. 12:3305–3312.

66. Cantrell, D.A. 1996. T cell antigen receptor signal transduction pathways. Annu. Rev. Immunol. 14:259–274.

67. Tang, T.L., R.M. Freeman, Jr., A.M. O'Reilly, B.G. Neel, and S.Y. Sokol. 1995. The SH2-containing protein tyrosine phosphatase SH-PTP2 is required upstream of MAP kinase for early xenopus development. Cell. 80:473–483.

68. Roche, S., J. McGlade, M. Jones, G.D. Gish, T. Pawson, and S.A. Courmidege. 1996. Requirement of phospholipase C-γ, the tyrosine phosphatase Syp and the adaptor proteins Shc and Nck for PDGF-induced DNA synthesis: evidence for the existence of Ras-dependent and Ras-independent pathways. EMBO (Eur. Mol. Biol. Organ.) J. 15:4940–4946.

69. Scharenberg, A.M., and J.-P. Kinet. 1996. The emerging field of receptor-mediated inhibitory signaling: SHP or SHIP? Cell. 87:961–964.