CTLA-4 Ligation Suppresses CD28-induced NF-κB and AP-1 Activity in Mouse T Cell Blasts*

Christina Olsson‡§, Kristian Riebeck‡, Mikael Dohlsten†, and Erik Michaelsson‡

From ‡Active Biotech Research AB and †Department of Medical Microbiology, Malmö University Hospital, and \Wallenberg Laboratory, Section of Tumor Immunology, Department of Cell and Molecular Biology, University of Lund, SE-220 07 Lund Sweden

The effects of cytotoxic lymphocyte antigen 4 (CTLA-4) on CD3/CD28 monoclonal antibody (mAb) activation of CD4⁺/CTLA-4⁺ blastoid T cells were studied in an in vitro model system. As previously reported, coligation of CTLA-4 mAb results in suppression of T cell proliferation and cytokine production. The proliferation but not the interleukin 2 (IL-2) production could be restored by addition of exogenous IL-2, suggesting that the inhibitory effect occurred at the level of IL-2 production rather than at the regulation of the IL-2 receptor pathway. To study the effects on nuclear factors critical for T cell activation, we analyzed the levels of the transcription factors NF-κB and AP-1. These were potently induced in CD3/CD28 mAb-restimulated T cells. In contrast, CTLA-4 ligation strongly suppressed the induction of both transcription factors. The compositions of NF-κB and AP-1 family members were similar, irrespective of stimulation conditions. Analyses of the NF-κB regulator IκB-α revealed similar levels of IκB-α protein in the preparations. However, a reduced phosphorylation of IκB-α in CTLA-4 coengaged T cell blasts compared with T cells ligated with CD3/CD28 was found. Previous studies have concluded that CTLA-4 ligation regulates T cell activation by inhibiting the T cell receptor-mediated signals. However, the present findings propose that the major impact of CTLA-4 ligation is inhibition of signals mediated by CD28.

The T cell surface receptors CD28 and CTLA-4 (CD152), and their ligands CD80 and CD86 on professional antigen-presenting cells, regulate the activation of T cell receptor (TCR)‐triggered T cells. CD28 is expressed on both resting and activated cells, whereas CTLA-4 is only detectable on activated cells (1, 2). The CD28/B7 pathway is known to be essential for the development and maintenance of T cell responses (3). CTLA-4 was originally thought to have a similar function as CD28. In agreement with this original thought, B7-dependent costimulation of CD28‐deficient T cells has been demonstrated, which suggests that CTLA-4 ligation results in costimulation (4). However, there is accumulating data showing that CTLA-4 coligation has a negative regulatory effect on T cells, such as down‐regulation of interleukin 2 (IL‐2) production and cell cycle progression (5, 6). This negative regulatory effect is supported not only by the phenotype of CTLA-4-deficient mice (7, 8) but also by the effects observed after administration of CTLA-4 monoclonal antibody (mAb) or Fab in animal models of autoimmune diseases and infection (6, 9–11).

The IL‐2 gene is regulated by signals mediated by TCR as well as CD28 signaling that ultimately leads to DNA binding of nuclear factors such as the prominent NF-κB and AP-1 transcription factors (12). After TCR stimulation, the immunoreceptor tyrosine‐based activation motifs within the TCR/CD3 complex interact with intracellular signaling molecules. The tyrosine residues within immunoreceptor tyrosine‐based activation motifs become phosphorylated and create binding sites for Src homology-containing proteins (13). The activation of these protein-tyrosine kinases results in phosphorylation of downstream substrates, e.g. p21ras/mitogen-activated protein kinases, phospholipase C, and phosphatidylinositol 3-kinase (14). This activation is attributable to tyrosine phosphorylation of the CD28 cytoplasmic domain after CD28 cross-linking. CD28/TCR signaling causes IκB-α phosphorylation, whereupon the IκB-α is degraded, and the IκB-α/NF-κB complex is resolved, allowing translocation of NF-κB into the nucleus (15, 16). Moreover, the mitogen-activated protein kinases extracellular signal-regulated kinase (ERK) and jun NH2-terminal kinase (JNK) are activated on signaling and induce c-Fos and c-Jun transcription and the following formation of AP-1 protein complexes (12).

Although the downstream consequences of CTLA-4 signaling are still unclear, two distinct models have been proposed to explain the inhibitory activity of CTLA-4 (17). The first model suggests that CTLA-4 antagonizes CD28 function, either by competing for B7 binding (2, 18, 19) and/or actively blocking CD28 signal transduction (5, 6, 20). The second model suggests that CTLA-4 interferes with TCR signaling. An effect on TCR signaling was recently proposed because of identification of hyperactive kinases associated with TCR observed in CTLA-4-deficient T cells (21), suggesting that CTLA-4 counteracts the hyperphosphorylation in a normal situation. Two recent studies using CD28-deficient mice found that CTLA-4 in the absence of CD28 antagonized the TCR-mediated signal, arguing that it is sufficient to explain the inhibitory effect of CTLA-4 (17, 22). A recent study using preactivated T cells showed that CTLA-4 selectively inhibited TCR induced ERK-2 activity and TCR-CD28-induced JNK activity (20). Furthermore, an intracellular activation motif in the CTLA-4 cytoplasmic domain has been identified, which shows homology to the CD28 cytoplasmic tail (24, 25). This common domain has been shown to be the...
binding site for p85 phosphatidylinositol 3-kinase; however controversy regarding its role in costimulation exists (26, 27).

In the present study, we examined the effect of CTLA-4 coligation on nuclear transcription factors in mouse CD4+ T cell blasts. We found that CTLA-4 ligation had profound effects on the transcription factors NF-κB and AP-1. Moreover, the effects of CTLA-4 ligation were only evident when CD28 was also coligated, supporting the hypothesis that the major function of CTLA-4 is suppression of CD28 signaling.

**EXPERIMENTAL PROCEDURES**

**Medium and Reagents—** RPMI 1640 medium supplemented with 2 mM l-glutamine, 0.10 mM HEPES, 1 mM NaHCO3, 0.1 mg/ml gentamicin sulfate, 1 mM sodium pyruvate, 10% fetal bovine serum, and 50 μM β-mercaptoethanol was used throughout for cell cultures.

UC10-4F10-11 (hamster IgG, anti-murine CTLA-4) hybridoma was a kind gift from Dr. J. Bluestone (University of Chicago, Chicago, IL). Anti-CD4-α mAbs were produced from the hybridoma and purified on a protein G column (Amersham Pharmacia Biotech).

Hamster anti-mouse CD3 (145-2C11), CD28-PE and unconjugated (37.51) CTLA-4-α mAbs (UC10-4F10-11), hamster IgG-PE/unconjugated (G235-2356, isotype control), and rat anti-mouse CD4-PE/fluorescein isothiocyanate (GK1.5) were purchased from PharMingen (San Diego, CA).

**Preparation of Mouse CD4+ T Cell Blasts—** Splenocytes from C57BL/6 mice (H-2b) purchased from M&B (Ry, Denmark) were used as a source of CD4+ T cells. Erythrocytes were depleted from spleen cell suspensions by hypotonic lysis, and the remaining cells were cultured in the presence of soluble anti-CD3 mAb (0.5 μg/ml). After 48 h, the cells were harvested and live cells were enriched by density centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech). The enrichment procedure was followed by positive selection using magnetic beads coated with anti-CD4 mAb (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer’s instructions. The purity of the CD4+ T cells (≥96%) was routinely checked by fluorescence-activated cell sorting (FACS) analyses.

Although not separated by size, they are denoted as T cell blasts throughout this paper for simplicity.

**T Cell Blast Proliferation and Cytokine Production—** Beads (Interfacial Dynamics Corp., Portland, Oregon) were coated with mAbs toward CD3, CD3/CD28, CD3/CTLA-4; CD3/CD28/CTLA-4; or hamster IgG (isotype control) as described by Krummel and Allison (5). The concentrations of mAbs used for coating were anti-CD3, 0.5 μg/ml; anti-CD28, 1 μg/ml; and anti-CTLA-4, 4.5 μg/ml. The total amount of mAb was compensated for by adding control mAb (trinitrophenol-specific hamster IgG) to a final Ig concentration of 6 μg/ml.

One hundred thousand CD4+ T cell blasts were cocultured with 105 coated beads in round-bottom 96-well plates (Nunc, Roskilde, Denmark). After 24 h of stimulation, the cells were pulsed for 4 h with 0.5 μCi of [3H]thymidine, and the amount of incorporated [3H]thymidine was determined by liquid scintillation counting (28). The amount of IL-2 was quantified by a sandwich enzyme-linked immunosorbent assay using specific Ab pairs from PharMingen (R4-62A and XM3G12). The amount of IFN-γ was quantified by a sandwich enzyme-linked immunosorbent assay using recombinant murine IFN-γ and specific Ab pairs from PharMingen (R4-62A and XM3G12).

**Preparation of Protein Extracts and Electrophoretic Mobility Shift Assay—** Nuclear and cytoplasmic protein extracts were prepared, with minor modifications, according to the method described by Schreiber et al. (29). Aliquots of protein were stored at −70 °C until required, and protein concentrations of extracts were measured with a Bio-Rad protein assay kit.

[1g32P]ATP-labeled probes (30,000 cpm) were prepared as described previously (30). The oligonucleotides used contained the following sequences: AP-1 consensus, 5′-CTAGTGATGATGATGCGCGATGTC-3′; NF-κB consensus, 5′-GATCGAGGAGGCCTTCCTAGC-3′; and Oct binding site, 5′-GCTCTGATGATGATGCGCGATGAAATCTCCGTGATGTC-3′ (31). Binding reactions were performed with the same amount of protein in each mixture (0.5–2 μg), and the samples were electrophoresed on a 6.5% polyacrylamide–Tris borate–EDTA gel as described previously (30). For supershift analyses, the mixture of nuclear protein extract and labeled oligonucleotide was incubated with 1 μg of Abs against various transcription factors for 1 h on ice. Polyclonal Abs directed to Rel family proteins p50 (sc-114), p65 (sc-372), c-Rel (sc-70), and c-Rel (sc-272, pan-c-Rel) and to AP-1 gene family proteins c-Jun/AP-1 (sc-44, pan-Jun), c-Jun/AP-1 (sc-45), Jun B (sc-046), Jun D (sc-74), c-Fos (sc-253), c-Fos (sc-052), Fra-1 (sc-183), and Fra-2 (sc-171) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Western Blot—** The cytoplasmic protein extracts were analyzed for content of total as well as phosphorylated IkB-α protein by Western blotting. Protein extracts were separated on 10% SDS-polyacrylamide gel electrophoresis and electrophorized onto nitrocellulose membranes (Millipore, Solna, Sweden). Phosphorylated IκB-α and total IκB-α protein were analyzed with the PhosphoPlus IkB-α antibody kit according to the instructions from the manufacturer (New England Biolabs, Beverly, MA).

**RESULTS**

**Coligation of CTLA-4 Down-regulates T Cell Proliferation and Cytokine Production—** Protein extracts were collected by positive selection according to magnetic cell separation and analyzed by FACS. Protein extracts were collected by positive selection according to magnetic cell separation and analyzed by FACS.

**T Cell Blasts Restimulated with CD28 Only did not show proliferation in response to rechallenge with CD28 only (Fig. 2). On the contrary, when CTLA-4 was coligated, T cell blasts did not produce IL-2 or proliferate, and IFN-γ production was reduced to the level observed for blasts restimulated with anti-CD3 only (Fig. 2). In cultures restimulated with control mAb only, none of the cytokines analyzed could be detected. The effects of CTLA-4 ligation on IL-2 production was also apparent at the mRNA level. At 40 and 90 min after restimulation, T cell blasts restimulated with anti-CD3/anti-CD28 showed a marked up-regulation of IL-2 mRNA, whereas the mRNA levels in T cell blasts restimulated with coligated CTLA-4 were comparable with the nonstimulated T cell blasts. Similar glyceraldehyde-3-phosphate dehydrogenase levels were detected in all samples (data not shown).

**T cell blast proliferation, but not the IL-2 production, could be restored by the addition of recombinant human IL-2 in cultures stimulated with coligated CTLA-4 (Fig. 2, A and C). Because the IL-2Rα expression was only slightly influenced by CTLA-4 coligation, this suggested that the influence of CTLA-4...**

---

**FIG. 1. CTLA-4 and CD28 expression on nonstimulated CD4+ T cells and CD4+ T cell blasts.** Spleen cells from C57BL/6 mice were stimulated with soluble anti-CD3 for 48 h, whereafter the CD4+ T cells were collected by positive selection according to magnetic cell separation and analyzed by FACS. A, nonstimulated CD4+ T cells; B, CD4+ T cell blasts. Dashed line, isotype control shown in gray, CTLA-4 expression; shown in black. CD28 expression. The graph is representative of three independent experiments.
was at the level of IL-2 protein production rather than regulation of the IL-2R pathway. These results are in agreement with others (6, 32).

CTLA-4 Ligation Abrogates Expression of the NF-κB and AP-1 Transcription Factors—We further dissected the impact of CTLA-4 ligation on intracellular signaling events by studying NF-κB and AP-1 binding activity in nuclear protein preparations from restimulated CD4+ T cell blasts. Nuclear protein extracts were analyzed using gel shift analysis and oligonucleotides encoding an NF-κB or an AP-1 consensus motif. The protein preparations from T cell blasts, restimulated with anti-CD3 mAb only, produced a weak signal of NF-κB and AP-1 binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3). The inclusion of CD28 mAb led to a substantial increase in both NF-κB-binding activity (Fig. 3).

We also analyzed DNA binding of the ubiquitous octamer binding proteins. Constitutive expression of two complexes, which most likely corresponded to Oct-1 and Oct-2, was seen in all nuclear extracts irrespective of stimulation conditions (Fig. 3).

Ligation of CD28 and CTLA-4 Induces Similar Composition of Either NF-κB or AP-1 Protein Complexes—The NF-κB/Rel family of transcription factors are composed of homo- or heterodimers of p50, p65, and c-Rel, whereas the AP-1 transcription factors are homo- or heterodimers of different members of the Fos (e.g. c-Fos, Fra-1, and Fra-2) and Jun (e.g. c-Jun, JunB, and JunD) family of proteins. Supershift analyses with Abs to a panel of different Rel, Fos, and Jun family proteins were used to determine whether the CTLA-4 inhibitory effect was attributable to differences in compositions of the prominent transcription factors NF-κB and AP-1. Because our findings propose that the major impact of CTLA-4 ligation is inhibition of signals mediated by CD28, we focused on studying T cell blasts stimulated with CD3/CD28 in the presence or absence of coligated CTLA-4.

The components of the Rel family of transcription factors revealed two complexes binding to the NF-κB element. The main constituent in T cell blasts stimulated with either mAb combination was a faster-migrating p50-p50 homodimer (Fig. 4A). Addition of Ab against p65 to nuclear protein preparations stimulated with either mAb combination reduced the upper complex, which indicated participation of p65 in the NF-κB-Rel complex. Because the upper complex was also shifted by Ab against p50, it is reasonable to assume that it represents a p50-p65 heterodimer. Also, supershift analyses of AP-1 components revealed a similar pattern irrespective of stimulation protocols, with the major Jun component present being Jun D. The AP-1 complex was reduced in intensity when Ab against p65 was added, indicating the participation of Fra-2 components in the AP-1 complexes (Fig. 4B). No shifts were observed with control IgG (data not shown). Control experiments performed with a mixture of Abs and DNA probe in the absence of protein extract showed that none of the Abs used bound directly to the DNA target sequences (data not shown).
Because a similar composition of either NF-κB or AP-1 family members appeared in the absence or presence of CTLA-4 coligation, it appears that CTLA-4-mediated down-regulation of T cell activation was attributable to interference with CD28 signal transduction rather than interference with a specific nuclear factor.

CTLA-4 Coligated T Cell Blasts Contain Low Levels of Phosphorylated IκB-α—NF-κB is present in the cytoplasm bound to the IκB-α protein; IκB-α retains NF-κB in the cytoplasm by masking the nuclear localization signal (16, 33). Phosphorylation of IκB-α at serines 32 and 36 leads to the release and nuclear translocation of active NF-κB/Rel transcription factors (16, 33) and represents the major regulatory step in NF-κB activation. Examination of cytoplasmic protein extracts by Western blot revealed that CD3/CD28 and CD3/CD28/CTLA-4 coligated T cell blasts contained similar levels of total IκB-α protein (Fig. 5A). Blotting with phosphospecific IκB-α Ab showed that CD4+ T cell blasts activated with CD3/CD28 mainly contained the slower-migrating phosphorylated form of IκB-α (Fig. 5B), which suggested that the majority of NF-κB has been translocated into the nucleus and is active in transcriptional events. Interestingly, cytoplasmic protein extracts from CTLA-4-colligated CD4+ T cell blasts demonstrated reduced levels of the phosphorylated IκB-α (Fig. 5B). The difference in phosphorylation status was more pronounced at 20 than at 4 h. Note that cytoplasmic protein extracts from T cell blasts that were not stimulated exhibited no detectable phosphorylated IκB-α protein (Fig. 5B), indicating that restimulation was required to induce phosphorylated IκB-α proteins.

DISCUSSION

It is well established that CD28 strongly costimulates T cells (3). Initially, it was assumed that a positive signal was also transduced through the CTLA-4 receptor. However, results have accumulated that suggest CTLA-4 mediates down-regulation of the ongoing immune response. It has been shown that cross-linked anti-CTLA-4 mAb inhibits proliferation in T cells stimulated with anti-CD3/anti-CD28 mAb (6, 9), whereas soluble anti-CTLA-4 mAb or Fab enhances T cell proliferation and cytokine production. The observations in CTLA-4 deficient mice have strengthened the hypothesis that a negative signal is mediated by CTLA-4 coligation (7, 8). In support of this, it has also been shown that costimulation with B7–1 inhibits the response of primed CD28-deficient T cells (17, 22). On the other hand, another study could demonstrate B7-dependent costimulation of CD28-deficient T cells, which suggested that CTLA-4 ligation led to T cell activation rather than down-regulation (4). These contradictory results indicate the importance of studying the effects of CD28 and CTLA-4 in a relevant model with both players present.

In attempts to identify intracellular targets involved in CTLA-4 signaling, a motif containing a consensus binding site of phosphatidylinositol 3-kinase was identified (26). Several studies have reported activation of a phosphatidylinositol 3-kinase (34, 35) and CTLA-4 ligation (36). However, the functional importance of the CD28-CTLA-4 interaction with phosphatidylinositol 3-kinase required for costimulation of T cells remains unclear (36). The tyrosine phosphatase SYP was demonstrated to specifically associate with the CTLA-4 receptor, and several reports have shown significant phosphatase activity toward the RAS regulator p21ras, indicating that the Ras pathway is regulated by CTLA-4 (21, Marengere and co-workers (21) were also able to show that CTLA-4 deficient T cells had hyperactivated protein-tyrosine kinases Fyn, Lck, and ZAP70. This argues for CTLA-4-negative regulation being mediated via the TCR signal pathway. That CTLA-4 ligation inhibits activation of CD28-deficient T cells has also been taken as an argument for CTLA-4 having its effect(s) on TCR signal-
ing-associated molecules. On the other hand, it cannot be ruled out that the CD28 signal pathway is the main target, and that the effects on TCR signaling represent additional substrates for CTLA-4 interference, which are revealed by the genetic removal of CD28.

In the present study, we examined the effects of CTLA-4 ligation in CD3/CD28-triggered CD4+ T cell blasts. The experimental model was designed to mimic the role of CTLA-4 on activated T cells. The rationale for doing the experiments on T cell blasts expressing both CD28 and CTLA-4 on the surface was that we believe that CTLA-4 exerts its main function by down-regulating these activated cells. We found a clear inhibitory effect on IL-2 mRNA induction, T cell growth, and cytokine production. Our results are in agreement with previous studies, which show a down-regulation of T cell activation by cross-linking CTLA-4 with CD3/CD28 (5). T cell blasts stimulated with CD3/CD28/CTLA-4 mAb and human recombinant IL-2 proliferated strongly, indicating that these cells expressed functional IL-2R. However, the cells produced no detectable IL-2, which suggested that the blockade of proliferation by CTLA-4 was mainly a consequence of its interference with IL-2 protein production.

Several transcription factors, such as NF-κB, AP-1, and Oct, have been described as critical for transcriptional activation of the IL-2 gene promoter (14, 37). Analysis of CTLA-4-ligated T cell blasts showed only small amounts of NF-κB and AP-1 binding activity in nuclear protein extracts. However, large amounts of NF-κB and AP-1 DNA binding activity were induced in CD3/CD28-stimulated T cell blasts. TCR signaling alone can lead to recruitment of NF-κB and AP-1; however, the magnitude of these factors is superinduced by CD28-mediated costimulation. In our studies, an inhibition of the analyzed transcription factors could not be observed after CTLA-4 and CD3 ligation alone but required CD28 ligation. Moreover, we found that coengagement of CTLA-4 induced only low levels of IκB-α phosphorylation in the cytosol, compared with a significant amount of phosphorylated IκB-α in CD3/CD28-stimulated T cell blasts. In contrast, similar levels of total IκB-α protein level were found in the cytoplasmic protein preparations. The existence of mainly unphosphorylated IκB-α may represent inactivation of residual low amounts of NF-κB in the cytosol of CTLA-4-ligated T cell blasts but could also be attributable to a CTLA-4-mediated reduction of synthesis of NF-κB/Rel family proteins.

Our findings concerning AP-1 are in concordance with a study by Calvo et al. (20), who showed that CTLA-4 selectively turns off activation of downstream TCR/CD28 signaling events, i.e. activation of ERK-2 and JNK (20). Interestingly, the effects shown on ERK-2 and JNK activity occurred very rapidly after stimulation, which is in contrast to the present results. In the present study a much later effect by CTLA-4 coligation was detected. Only small quantitative differences were exhibited at present study a much later effect by CTLA-4 coligation was detected. Only small quantitative differences were exhibited at

REFERENCES
Acids Res. 17, 6419
30. Sundstedt, A., Sigvardsson, M., Leanderson, T., Hedlund, G., Kalland, T., and Dohlsten, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 979–984
31. Jain, J., Miner, Z., and Rao, A. (1993) J. Immunol. 151, 837–848
32. Finn, P. W., He, H., Wang, Y., Wan, Z., Guan, G., Listman, J., and Perkins, D. L. (1997) J. Immunol. 158, 4074–4081
33. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) Science 267, 1485–1488
34. Hutchcroft, J. E., Franklin, D. P., Tsai, B., Harrison-Findik, D., Varticovski, L., and Bierer, B. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8808–8812
35. Pages, F., Ragueneau, M., Rottapel, R., Truneh, A., Nunes, J., Imbert, J., and Olive, D. (1994) Nature 369, 327–329
36. Schneider, H., Prasad, K. V., Shoelson, S. E., and Rudd, C. E. (1995) J. Exp. Med. 181, 351–355
37. Serfling, E., Avots, A., and Neumann, M. (1995) Biochim. Biophys. Acta 1263, 181–200
38. Lee, F. S., Peters, R. T., Dang, L. C., and Maniatis, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9319–9324