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CTLA-4 (CD152) Can Inhibit T Cell Activation by Two Different Mechanisms Depending on Its Level of Cell Surface Expression

Beatriz M. Carreno,* Frann Bennett,* Thu A. Chau,† Vincent Ling,* Deborah Luxenberg,* Jason Jussif,* Miren Lorea Baroja,‡ and Joaquin Madrenas†

CTLA-4 (CD152) engagement results in down-regulation of T cell activation. Two mechanisms have been postulated to explain CTLA-4 inhibition of T cell activation: negative signaling and competitive antagonism of CD28:B7-mediated costimulation. We assessed the contributions of these two mechanisms using a panel of T cell lines expressing human CTLA-4 with mutations in the cytoplasmic region. Under conditions of B7-independent costimulation, inhibition of IL-2 production following CTLA-4 engagement was directly proportional to CTLA-4 cell surface levels and did not require its cytoplasmic region. Thus, CTLA-4 down-regulates T cell activation by two different mechanisms—delivery of a negative signal or B7 sequestration—that are operational depending on the levels of CTLA-4 surface expression. These two mechanisms may have distinct functional outcomes: rapid inhibition of T cell activation or induction of T cell anergy. The Journal of Immunology, 2000, 165: 1352–1356.

**Materials and Methods**

**Plasmids and Abs**

Human CTLA-4 (hCTLA-4) cDNA was obtained from G. Freeman (Dana-Farber Cancer Institute, Boston, MA). Mutant CTLA-4 cDNAs were generated using the Chameleon site-directed mutagenesis kit (Stratagene, La Jolla, CA) or PCR amplification with high fidelity KlenTaq polymerase (Clontech Laboratories, Palo Alto, CA), and the introduced mutation was confirmed by DNA sequencing. cDNAs were subcloned into the EcoRI site of pBIG2i, a vector that utilizes a hybrid bidirectional tetracycline-responsive promoter element to direct expression of both the CTLA-4 cDNA as well as the rtAN tetracycline-responsive transactivator (10). mAbs against human CTLA-4 are anti-CTLA-4-38 (murine IgG1 mAb that blocks CD80 and CD86 binding to CTLA-4), and anti-CTLA-4-33 (murine IgG1 mAb that does not block CD80 or CD86 binding to CTLA-4).

**CTLA-4 cell lines**

Stable Jurkat T cell transfectants were generated as described (10). Briefly, \(5 \times 10^6\) Jurkat E6.1 cells were transfected by electroporation (300 V and 950 μF capacitance using a gene pulser (Bio-Rad, Hercules, CA)) with 10 μg linearized plasmid DNA from the different pBIG2i constructs. Stable transfectants were selected with hygromycin (Life Technologies, Gaithersburg, MD). Results with a representative clone are shown. CTLA-4 expression was induced by overnight incubation with the indicated concentration of doxycycline (Sigma, St. Louis, MO).

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*Abbreviations used in this paper: h, human; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; hCTLA-4, human CTLA-4; WT, wild type; TL, tailless.*
**T cell functional assays**

Anti-CD3 (1 μg/10^7 beads, UCHT1; PharMingen, San Diego, CA) or anti-CD28 mAb (5 μg/10^7 beads, PharMingen) mAb-coated latex beads (4 μg/10^7 beads, Dynal, Lake Success, NY) were prepared as described (15). Anti-CD3 (1 μg/10^7 beads) mAb/hB7.2-Ig (4 μg/10^7 beads)-coated latex beads (Interfacial Dynamics, Portland, OR) were prepared as described (16). Ab-coated beads were added to untreated or doxycycline-induced Jurkat cells in the presence or absence of soluble anti-CD28 mAb (5 μg/ml; CD28.2, PharMingen). Supernatants were harvested at 48 h, and IL-2 was measured using an ELISA kit (Genzyme Diagnostics, Framingham, MA).

**CTLA-4 biochemistry**

Lysates from doxycycline-treated (100 ng/ml) cells were prepared and CTLA-4 expression was monitored by immunoblotting using an anti-CD28 mAb (CTLA-4-20A) or control (anti-HLA class I; PharMingen) mAb-coated tosyl beads (4 μg/10^7 beads)-coated latex beads (Interfacial Dynamics, Portland, OR) were prepared as described (17). Phosphorylated extracellular signal-regulated kinase-1 (ERK-1) and ERK-2 were detected using an anti-active mitogen-activated protein kinase (MAPK) rabbit antiserum (Promega, Madison, WI). Blots were reprobed with a rabbit antiserum to total MAPK/ERK-1-CT (18). Signal quantitation was performed with an imaging densitometer (GS 700, Bio-Rad) and Molecular Analyst software (version 1.0, Bio-Rad).

**Results and Discussion**

We previously described a panel of Jurkat T cell lines expressing human wild-type (WT) or mutant CTLA-4 molecules under the control of a doxycycline-inducible promoter (10). In this paper, we expand the structure-function analysis of CTLA-4 using two activation systems: 1) a B7-independent system, consisting of anti-CD3/anti-CTLA-4 mAb-coated beads in the presence of soluble anti-CD28 mAb, and 2) a B7-dependent system, consisting of anti-CD3 mAb/hB7.2-Ig beads.

Parental Jurkat E6.1 cells with or without doxycycline treatment produced similar levels of IL-2 when stimulated with anti-CD3 mAb-coated or anti-CD3/anti-CTLA-4 mAb-coated beads in the presence of soluble anti-CD28 mAb (Fig. 1A). Thus, nontransfected Jurkat cells do not express functional CTLA-4. WT CTLA-4-expressing Jurkat T cells treated with or without doxycycline produced similar levels of IL-2 upon activation with anti-CD3 mAb-coated beads plus soluble anti-CD28 mAb. In contrast, activation with anti-CD3/anti-CTLA-4 mAb-coated beads resulted in a significant decrease (~85% inhibition) in IL-2 production in doxycycline-treated cells (Fig. 1B). Similarly, activation of doxycycline-treated, but not untreated, WT CTLA-4-expressing cells with anti-CD3/B7.2-Ig-coated beads inhibited IL-2 production by ~56% (Fig. 1C). Furthermore, engagement of CTLA-4 by either B7.1 or B7.2 on doxycycline-treated WT CTLA-4-expressing cells leads to down-regulation of IL-2 production (data not shown). Thus, using these two activation systems (anti-CD3/anti-CTLA-4 mAb beads or anti-CD3 mAb/hB7.2-Ig beads) in conjunction with mutant CTLA-4 molecules, we can mechanistically differentiate between CTLA-4-mediated signaling and competition between CD28 and CTLA-4 for B7.

Recently, several reports (9, 11) have examined whether the cytoplasmic tail of CTLA-4 is required for down-regulation of T cell responses. However, these studies were limited because either the contribution of endogenous CTLA-4 molecules could not be excluded (9) or the cytoplasmic tail was not fully truncated (9, 11). Since we have already reported that cytoplasmic tyrosine residues are not required for CTLA-4-mediated negative signaling (10), we concentrated on the cytoplasmic proline residues and the whole cytoplasmic region. Jurkat T cell lines expressing doxycycline-inducible CTLA-4 molecules with mutations on proline residues 169 and 173 (potential Src homology-3 domain-binding sites), or tailless (TL) CTLA-4 molecules truncated at residue 153 were generated (Fig. 2). To determine the ability of these molecules to deliver a negative signal, doxycycline-induced cells were activated with anti-CD3 mAb- or anti-CD3/anti-CTLA-4 mAb-coated beads and IL-2 production was assessed at 48 h. Activation with anti-CD3/anti-CTLA-4 beads inhibited IL-2 production equivalently on WT, and proline-deficient CTLA-4-expressing cells (Fig. 3A, top). Thus, proline residues 169 and 173 are dispensable for CTLA-4 signaling. In contrast, production of IL-2 was minimally affected by CTLA-4 engagement on cells expressing TL CTLA-4 molecules (Fig. 3A, bottom), and this was observed in spite of a 100-fold increase in TL CTLA-4 expression upon doxycycline treatment. Furthermore, these results correlated with the lack of...
inhibition of TCR-induced ERK activation by TL CTLA-4 molecules (Fig. 3B) (10, 19, 20), even after correction for levels of total ERK expressed by different transfectant clones. Based on these results we conclude that the cytoplasmic tail of CTLA-4, but neither its tyrosine (10) nor proline residues, is required for delivery of a signal that leads to inhibition of T cell activation.

The inability of TL CTLA-4 molecules to deliver a negative signal provided the unique opportunity to test whether competition between cell surface CTLA-4 and CD28 for B7 plays a role in the inhibition of T cell responses. Additionally, the ability to control the levels of CTLA-4 cell surface expression using different concentrations of doxycycline allowed us to examine the quantitative effect of CTLA-4:CD28 ratios on the outcome of T cell activation. As shown in Fig. 4A, noninduced TL CTLA-4-expressing cells produced IL-2 upon activation with anti-CD3/B7.2-Ig beads. As these cells expressed minimal CTLA-4 levels on their surface, IL-2 production was not affected by the addition of blocking (CTLA-4-38) or nonblocking (CTLA-4-33) anti-CTLA-4 mAbs. In contrast, upon maximal CTLA-4 expression, activation with anti-CD3 mAb/B7.2-Ig beads resulted in down-regulation of IL-2 production. Addition of an Ab (CTLA-4-38) capable of blocking CTLA-4:B7 interaction was able to restore IL-2 production to levels observed in noninduced cells (Fig. 4A). This provides the first demonstration that CTLA-4 molecules unable to deliver an intracellular signal can still inhibit IL-2 production by B7 sequestration. This effect was proportional to the levels of CTLA-4 expression on

![FIGURE 3. Inhibition of IL-2 production by CTLA-4-mediated signaling requires its cytoplasmic domain but not its proline residues. A, Doxy+ transfectants were stimulated with anti-CD3 mAb- or anti-CD3/anti-CTLA-4 mAb-coated beads in the presence of soluble anti-CD28 (5 μg/ml) as described in Fig. 1. Results are those obtained with cells treated with 100 ng/ml doxycycline; similar results are obtained when cells are treated with different doxycycline concentrations to achieve similar levels of CTLA-4 expression. Supernatants were harvested at 48 h, IL-2 production was determined by ELISA, and values were normalized. A value of 1 equals IL-2 amounts produced by anti-CD3-stimulated samples. *p < 0.05 compared with anti-CD3-stimulated samples.](http://www.jimmunol.org/)

![FIGURE 2. Expression of CTLA-4 molecules. Jurkat cells transfected with WT, Pro- (residues 169 and 173, Pro to Ala) or TL (truncated at residue 153) CTLA-4 cDNAs were cultured overnight with doxycycline (100 ng/ml). Lysates were analyzed by SDS-PAGE and immunoblotted with an anti-CTLA-4 mAb. A comparison of the cytoplasmic tail of the various molecules is presented.](http://www.jimmunol.org/)
Our report presents the first formal demonstration that competition between cell surface CTLA-4 and CD28 can occur. In contrast to negative signaling, B7 sequestration by CTLA-4 does not require CTLA-4 cytoplasmic tail, and is instead regulated by the levels of surface expression of CTLA-4. The finding that cell surface CTLA-4 can compete with CD28 for B7 binding explains why the expression of CTLA-4 is tightly regulated and highly compartmentalized (22), because high levels of cell surface CTLA-4 expression would lead to an inability to mount or expand T cell response.

The two mechanisms of CTLA-4 action may be operational in different biological contexts. We predict that in early stages of an immune response, under conditions of limited B7 and CTLA-4 expression, CTLA-4-mediated negative signaling would be the primary mechanism for inhibition of T cell activation. In contrast, at late stages on the immune response when there is increased B7 and CTLA-4 expression, both negative signaling through CTLA-4 and B7 sequestration would be operational.

Finally, the two different mechanisms of CTLA-4 action may ultimately cause different downstream effects. On the one hand, CTLA-4-mediated negative signaling will rapidly inhibit T cell activation. On the other hand, CTLA-4-mediated B7 sequestration will limit CD28-mediated signaling and lead to T cell anergy. This may provide a mechanistic basis for the involvement of CTLA-4 in peripheral tolerance (23, 24).

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References

FIGURE 4. CTLA-4 inhibits CD28-mediated T cell activation by sequestering B7. A. Doxy+ or doxy- TL CTLA-4-expressing cells were activated with anti-CD3 mAb/B7.2-Ig-coated beads in the CTLA-4 TL-expressing cells treated with various concentrations of doxycycline. Results are representative of three experiments. B. The expression of IL-2 production obtained at the indicated doxycycline concentrations was assessed by FACS analysis. Mean fluorescence values for CD28 expression relative to IL-2 levels obtained with untreated cells. CTLA-4 expression of IL-2 production was determined by ELISA. Results are presented as the percent production normalized to untreated and 100 ng/ml doxycycline-treated cells, respectively. Results are representative of three experiments.

the T cell surface (Fig. 4B). In the presence of an antagonistic anti-CTLA-4 mAb, production of IL-2 was restored to that seen in noninduced T cells (Fig. 4B).

Our results demonstrate that CTLA-4 can down-regulate T cell responses by two different mechanisms. One mechanism is negative signaling as shown here and previously reported (9–11, 20). We show that this mechanism requires the cytoplasmic tail of CTLA-4, emphasizing the importance of residues other than tyrosine or proline in negative signaling through CTLA-4. The other mechanism is cell surface competition with CD28 for B7 ligation. Although a soluble chimeric CTLA-4 fusion protein (CTLA-4.Ig) has been used as an immunosuppressive agent (21), this does not necessarily imply that membrane CTLA-4 should sequester B7.


