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

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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.

Cytolytic T lymphocyte-associated Ag-4 plays a critical role in the down-regulation of T cell responses, T cell homeostasis, and maintenance of peripheral tolerance as illustrated by the deleterious effects observed in CTLA-4-deficient mice (1–3). Two mechanisms have been postulated to mediate CTLA-4 inhibition of T cell responses. One mechanism for CTLA-4 function emphasizes negative signaling through this molecule upon TCR activation. A putative Src homology-2 domain binding sequence, centered at tyrosine residues 165 and 182, is present in the cytoplasmic tail of CTLA-4 (4). Although phosphorylation of these residues (particularly tyrosine 165) is important for retention of CTLA-4 on the cell surface (5–8), it is not required for CTLA-4-mediated inhibition of T cell activation (9–11). This implies that negative signaling through CTLA-4 may involve phosphotyrosine-independent mechanisms. Moreover, recent data have questioned the role of the cytoplasmic tail in CTLA-4 function (9, 11). Thus, the contribution of and the structural requirements for CTLA-4 signaling to inhibit T cell responses remain unclear.

A second mechanism proposes that CTLA-4 inhibition of T cell activation is due to antagonism of CD28-mediated costimulation. Both CD28 and CTLA-4 share the ligands B7.1 (CD80) and B7.2 (CD86) (4); however, the affinity of the CTLA-4:B7 interaction is 10 times higher than that of the CD28:B7 interaction (12). Although studies using CD28-deficient mice have shown that negative signaling through CTLA-4 is independent of CD28 expression (13, 14), it is plausible that CTLA-4 interacts with B7 and prevents its interaction with CD28. Thus, a mechanism by which CTLA-4 sequesters B7 could still be operational under conditions in which B7 expression is limiting.

To establish the contribution of these two mechanisms to CTLA-4-mediated inhibition of T cell activation, a panel of Jurkat T cell lines expressing mutant CTLA-4 molecules were activated with anti-CD3 mAb in the presence of either anti-CTLA-4 mAb or B7 molecules.

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 mutations were confirmed by DNA sequencing. cDNAs were subcloned into the EcoRI site of pBHG2i, a vector that utilizes a hybrid bidirectional tetracycline-responsive promoter element to direct expression of both the CTLA-4 cDNA as well as the rtTAN 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-4-43 (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 × 10⁶ 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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T cell functional assays

Anti-CD3 (1 μg/10^7 beads, UCHT1; PharMingen, San Diego, CA)/anti-CTLA-4 (CTLA-4-20A), or control (anti-HLA class I; PharMingen) mAb-coated toy1 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-CTLA-4 mAb (CTLA-4-11) (10). Doxycycline-treated cells were stimulated with anti-CD3- or anti-CD3/anti-CTLA-4 mAb-coated beads for 10 min. Cell lysates were prepared and immunoblotted 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/hB7.2-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...
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.

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 a 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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