Cutting Edge: Tyrosine-Independent Transmission of Inhibitory Signals by CTLA-4

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Engagement of CTLA-4 (CD152) on the surface of activated T lymphocytes inhibits expression of activation Ags, cytokine production, and proliferation. In vivo, a component of CTLA-4-mediated inhibition can be attributed to successful competition with the costimulatory receptor CD80 for binding to their shared natural ligands, B7-1 (CD80) and B7-2 (CD86). However, studies of cross-linking CTLA-4 by mAbs demonstrate that CTLA-4 also transduces inhibitory signals to T cells (1, 2).

The intracellular domain of CTLA-4 is highly conserved between species and has two Tyr residues. Tyr201 (murine sequence) is part of a motif (Tyr-Val-Lys-Met) that controls localization and trafficking of CTLA-4 (3). In the unphosphorylated state, this motif binds to AP-1 and AP-2 clathrin adaptor complexes that target CTLA-4 to intracellular compartments. Phosphorylation of Tyr201 releases the adaptor complexes and increases cell surface expression of CTLA-4 (4–8).

The proximal events involved in inhibitory signaling by CTLA-4 have yet to be fully elucidated. When phosphorylated, the Tyr-Val- Lys-Met motif binds the p85 subunit of phosphatidylinositol 3’-kinase (PI3K) (9), but the functional significance of this association is uncertain. CTLA-4 also can associate with the tyrosine phosphatase SHP-2 (SH2 domain-bearing protein tyrosine phosphatase). Several studies indicate that phosphorylation of Tyr201 recruits SHP-2 to CTLA-4 (10–12), but one recent report observed tyrosine-independent association of CTLA-4 and SHP-2 (13). CTLA-4-associated SHP-2 has been reported to dephosphorylate the ζ-chain of the TCR complex (13) or to inhibit the TCR signaling pathway by dephosphorylating several of its other key components: Fyn, Lck, ZAP-70, and the Ras regulator p52SHC (10). However, not all studies have found an inhibition of early TCR signaling events. Revilla Calvo et al. (14) did not observe an effect of CTLA-4 ligation on the phosphorylation of TCR ζ and ZAP-70 but did find inhibition of more downstream events: the activation of extracellular signal regulated kinase and Jun N-terminal kinase.

To explore the nature of CTLA-4 signaling and to define its structural requirements, we studied the effects of cytoplasmic domain mutations on the ability of CTLA-4 to inhibit IL-2 production by Jurkat T cells stimulated through CD3 and CD28. Our results demonstrate that tyrosine phosphorylation of CTLA-4 is not necessary for inhibitory signaling in this system and establish an important role for the membrane-proximal region of the cytoplasmic domain.

Materials and Methods

Abs and reagents
cDNA encoding wild-type (WT) murine CTLA-4 (mCTLA-4) was provided by Dr. Peter Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). UC10-4F10-11 (hamster anti-mCTLA-4 mAb) hybridoma was a gift of Dr. Jeffrey Bluestone (University of Chicago, Chicago, IL). Purified mAb was obtained from ascitic fluid using protein A agarose (Life Technologies, Gaithersburg, MD). Control hamster mAb G235-2356 (anti-trinitrophenol), and murine mAbs UCHT1 and CD28.2 specific for human CD3ε and CD28, respectively, were purchased from PharMingen (San Diego, CA).

Generation and expression of mCTLA-4 mutants
All mutant cDNAs were generated from WT mCTLA-4 by established PCR methods as described previously (15) and confirmed by double-stranded sequencing. The mutants were subcloned into the expression vector pBSRαEN and stably transfected into Jurkat E6.1 cell line (Ref. 15; Fig. 1).

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3 Abbreviations used in this paper: SHP-2, SH2 domain-bearing protein tyrosine phosphatase; mCTLA-4, murine CTLA-4; WT, wild type.
The beads were then resuspended at the density of 2 x 10^7 beads/ml were incubated in PBS containing 4 µg/ml of anti-human CD3 mAb and 20 µg/ml of anti-mouse CTLA-4 mAb at 37°C for 1.5 h. The unoccupied binding sites were blocked by 2% BSA in PBS for 30 min at room temperature, followed by two washes with complete RPMI medium (RPMI 1640 with glutamine supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM 2-ME, all from Life Technologies). The beads were then resuspended at the density of 2 x 10^7 beads/ml in the same medium, and the exact number was verified by counting with Coulter Counter ZBI (Coulter Electronics, Hialeah, FL). The control beads were coated with anti-human CD3 mAb and control hamster IgG in the same way except the ratio of coating mAbs was adjusted to compensate for differences in binding capacities between hamster IgG and anti-mouse CTLA-4 mAb. This was done to obtain approximately equal stimulation of untransfected cells by the same number of anti-CD3/mCTLA-4 and anti-CD3/control beads. Usually several batches of slightly differing control beads were prepared and the best matching batch was used for experiments.

**Results and Discussion**

Following its induction on activated T cells, the cell surface expression of CTLA-4 is limited and tightly regulated, with the majority of CTLA-4 localized to intracellular stores. Similarly, after stable transfection with a cDNA encoding WT mCTLA-4, Jurkat T cells expressed only low levels of mCTLA-4 on the cell surface, as detected by staining on ice with an anti-mCTLA-4 mAb (Fig. 2A). However, staining at 37°C revealed considerably higher levels of cellular mCTLA-4 (Fig. 2B). The enhanced staining at 37°C is consistent with the behavior of CTLA-4 in activated T cells and likely reflects trafficking of mCTLA-4 between the cell surface and intracellular stores, with the consequent intracellular accumulation of the staining mAb (16).

Jurkat cells produce readily detectable levels of IL-2 in response to the combination of anti-CD3 and anti-CD28 mAbs. To determine whether mCTLA-4 can deliver an inhibitory signal to Jurkat, we stimulated Jurkat cells with varying amounts of beads coated either with anti-CD3 mAb plus anti-CTLA-4 mAb (anti-CD3/CTLA-4 beads) or with anti-CD3 mAb plus a control hamster mAb (anti-CD3/control beads). Soluble anti-CD28 mAb was added to all cultures to provide costimulation.

As expected, untransfected Jurkat cells produced comparable levels of IL-2 in response to the two types of beads (Fig. 3A). In marked contrast, WT mCTLA-4-expressing Jurkat cells secreted substantially lower amounts of IL-2 when stimulated with anti-CD3/CTLA-4 beads than with anti-CD3/control beads (Fig. 3B). The differences were reproducible and significant (see below, Fig. 5). The inhibitory effect of anti-mCTLA-4 was most pronounced at the smallest doses of beads, suggesting that greater engagement of CD3 molecules overcomes the inhibitory signaling of mCTLA-4. Consistent with the findings of Fraser et al. (17), inhibition required immobilization of the anti-CD3 and anti-CTLA-4 mAbs on the same bead. When we performed these experiments using a mixture of beads coated separately with anti-CD3 and anti-mCTLA-4 mAbs, we observed either no effect of anti-mCTLA-4 or a slight increase in IL-2 production (data not shown).

To determine whether the inhibitory effect on IL-2 production requires the mCTLA-4 cytoplasmic domain, we created a truncation mutant (TR2) that deletes all but 6 aa of the intracellular...
This truncation removes the motif responsible for the intracellular localization and trafficking of CTLA-4 (3). As expected with deletion of that motif, the staining of TR2 was bright and was comparable on ice and at 37°C (Fig. 2, C and D), indicating that the great majority of TR2 was expressed on the cell surface. In marked contrast to WT mCTLA-4-expressing Jurkat cells, TR2-expressing cells produced more IL-2 in response to anti-CD3/CTLA-4 beads than anti-CD3/control beads (Fig. 4). The levels of cell surface TR2 were considerably higher than the levels of WT mCTLA-4 expressed by Jurkat cells (Fig. 2, A and C). To be certain that TR2 does not transmit inhibitory signal when expressed on the cell surface at levels comparable to those of WT mCTLA-4, we isolated a clone with low expression of TR2 (Fig. 2, E and F). The enhanced stimulatory effect of the anti-CD3/CTLA4 beads was less pronounced with this clone but was still apparent (Fig. 4, TR2LOW). Thus, either TR2 transmits a positive signal, or, more likely, its expression increases the avidity of the interaction between Jurkat cells and the beads, thereby facilitating engagement of CD3. In either event, however, the ability of mCTLA-4 to inhibit IL-2 production clearly depends upon sequences that are deleted by the TR2 truncation.

To determine whether CTLA-4 signaling requires its two cytoplasmic Tyr residues, we created a mutant (ALL F mCTLA-4) in which both Tyr201 and Tyr218 are replaced with Phe (Fig. 1). Mutation of Tyr201 also affects the motif involved in the intracellular localization and trafficking of mCTLA-4, and the ALL F mutant was expressed at higher levels on the cell surface than WT mCTLA-4 (Fig. 2, G and H). Mutation of the two cytoplasmic Tyr had little effect on the ability of mCTLA-4 to transmit an inhibitory signal (Fig. 5). At low doses of beads, the inhibitory capacity of ALL F was comparable to that of WT mCTLA-4 (p > 0.1 for 5 and 10 µl of anti-CD3/CTLA-4 beads). At higher doses of the anti-CD3/CTLA-4 beads, ALL F continued to inhibit, but not as effectively as WT mCTLA-4.

Pro-X-X-Pro motifs can interact with Src homology 3 domains and constitute a Tyr-independent mechanism for the recruitment of
signaling molecules. The cytoplasmic domain of mCTLA-4 contains one Pro-X-X-Pro sequence. To examine the possible significance of that sequence, we generated a Jurkat clone that expressed a truncation mutant of mCTLA-4 (TR1) that deleted the carboxy-terminal half of the cytoplasmic domain, including the Pro-X-X-Pro sequence (Fig. 1). The cell-surface expression of TR1, which retains the motif responsible for intracellular localization and trafficking, was comparable to that of WT mCTLA-4 (Fig. 2, I and J). Deletion of the distal half of the intracellular region had no significant effect on the ability of mCTLA-4 to transmit an inhibitory signal when the lower doses (5 or 10 μl) of the anti-CD3/CTLA-4 beads were used (Fig. 5). As was observed with the ALL F mutant, TR1 was less effective than WT CTLA-4 with higher doses of the beads.

Our results demonstrate that engagement of mCTLA-4 by mAb down-regulates the production of IL-2 by Jurkat cells stimulated through CD3 and CD28. The inhibitory signal delivered by mCTLA-4 in this system requires neither cytoplasmic Tyr residues nor the carboxy-terminal half of the cytoplasmic domain. The ALL F and TR1 mutants appear somewhat less efficient in inhibiting IL-2 than WT CTLA-4. These mutations may alter the overall structure of the molecule so that signaling is less efficient or may affect directly sites for coupling to signaling pathways. Regardless, however, the more striking finding is the extent to which inhibitory signaling is preserved despite mutation of all tyrosines or deletion of the carboxy-terminal half of the cytoplasmic domain.

Our findings argue against the paradigm of tyrosine-dependent recruitment of signaling molecules to CTLA-4 but are in accord with the recent finding that tyrosine phosphorylation is not required for CTLA-4 to associate with SHP-2 and TCR (13). Despite the presence of readily detectable levels of SHP-2 in Jurkat and despite considerable effort, we have not been able to demonstrate coinmunoprecipitation of SHP-2 with WT mCTLA-4 in these cells. This may simply reflect technical difficulties, but we cannot exclude the alternative possibility that, in Jurkat, mCTLA-4 signaling is independent of stable interactions with SHP-2.

The findings here present a sharp contrast with similar studies of CD28. Despite considerable sequence similarities, the two molecules appear to use distinct regions of their cytoplasmic domains for signaling. Tyr201, Tyr218, and the two Pro residues of the Pro-X-Pro sequence in CTLA-4 are conserved in CD28, but, unlike CTLA-4, each of these appears important for CD28 signaling (18–21). In contrast to the preservation of CTLA-4 function observed with TR1, the analogous truncation of CD28 eliminates costimulation (18). Comparison of our two mCTLA-4 truncation mutants suggests a critical role for the membrane-proximal region spanning residues 194–204. Deletion of this region, which occurs with truncation from TR1 to TR2, completely abrogates delivery of an inhibitory signal by mCTLA-4. Thus, these 11 aa either are directly involved in signal transduction or are required for the structural integrity of a more proximal signaling region.

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