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Hierarchical Regulation of CTLA-4 Dimer-Based Lattice Formation and Its Biological Relevance for T Cell Inactivation

Peter J. Darlington, Mark G. Kirchhof, Gabriel Criado, Jitin Sondhi, and Joaquín Madrenas

CTLA-4 is an activation-induced, homodimeric inhibitory receptor in T cells. Recent crystallographic reports have suggested that it may form lattice-like arrays on the cell surface upon binding B7.1/B7.2 (CD80, CD86) molecules. To test the biological relevance of these CTLA-4-B7 lattices, we introduced a C122A point mutation in human CTLA-4, because this residue was shown to be essential for dimerization in solution. Surprisingly, we found that up to 35% of C122A CTLA-4 dimerized in human T lymphocytes. Moreover, C122A CTLA-4 partitioned within lipid rafts, colocalized with the TCR in the immunological synapse, and inhibited T cell activation. C122-independent dimerization of CTLA-4 involved N-glycosylation, because further mutation of the N78 and N110 glycosylation sites abrogated dimerization. Despite being monomeric, the N78A/N110A/C122A triple mutant of CTLA-4 localized in the immunological synapse and inhibited T cell activation. Such functionality correlated with B7-induced dimerization of these mutant molecules. Based on these data, we propose a model of hierarchical regulation of CTLA-4 oligomerization by which B7 binding ultimately determines the formation of dimer-dependent CTLA-4 lattices that may be necessary for triggering B7-dependent T cell inactivation. The Journal of Immunology, 2005, 175: 996–1004.

T cell activation is regulated by a complex balance of co-stimulatory and inhibitory signals (1). One of the latter types of signals is delivered by CTLA-4 (CD152), an activation-induced transmembrane receptor expressed by T cells (2, 3). The ligands for CTLA-4 are B7.1 (CD80) and B7.2 (CD86), the same ligands as those for the costimulatory receptor CD28 (4). However, CTLA-4 binds to B7 molecules with a higher affinity and avidity compared with CD28. This allows CTLA-4 to sequester B7 ligands from CD28 and antagonize CD28-dependent costimulation (5, 6). In addition, CTLA-4 can inhibit T cell activation by initiating a signal transduction pathway that leads to inhibition of TCR-dependent signaling (reviewed in Ref. 7). This latter mechanism involves accumulation of CTLA-4 in lipid rafts, where it may exert its down-regulatory function on signaling initiated from TCR subunits (8–10). CTLA-4 also colocalizes with the TCR in the immunological synapse (IS), where it may inhibit TCR-proximal signaling (8, 9, 11).

An unresolved question regarding the biology of CTLA-4 is how ligation of this receptor by B7 molecules triggers inhibitory signals for T cell activation. Classical receptor signaling models claim that signaling either results from ligand-induced conformational changes, as in the case of the insulin receptor (12), or, alternatively, results from ligand-induced dimerization, as in the case of human growth hormone (13). However, CTLA-4 does not undergo any detectable conformation change upon B7 binding (14), and before ligation, it exists as a nonfunctional covalent homodimer (15). Therefore, alternative models are currently being explored to explain CTLA-4 signaling.

Based on the recent crystal structure of CTLA-4 and B7, it has been suggested that CTLA-4 homodimers form periodic arrays, or lattices, with homodimeric B7 (14, 16). Such lattices are postulated to provide the appropriate oligomerization conditions for CTLA-4 signaling to occur (17, 18). Because CTLA-4 coclusters with the TCR in the IS (8, 11), such lattices would be poised to inhibit TCR/CD28-mediated T cell activation. To test the hypothesis that dimer-dependent CTLA-4-B7 lattices facilitate the function of CTLA-4, we analyzed the function of CTLA-4 molecules containing a cysteine to alanine point mutation at position 122 (C122A). This cysteine residue has been shown to mediate intermolecular disulfide bonding in the CTLA-4 dimer and is critical for CTLA-4 dimerization in solution (15). Surprisingly, when a C122A mutation was introduced, up to 35% of CTLA-4 was dimeric. Furthermore, C122A CTLA-4 compartmentalized in lipid rafts, migrated to the IS upon stimulation where it colocalized with the TCR, and inhibited T cell activation. C122A CTLA-4, which underwent aberrant N-glycosylation, failed to dimerize when N78 and N110

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Abbreviations used in this paper: IS, immunological synapse; ER, endoplasmic reticulum; LAT, linker for activation of T cell; NNC, N78A/N110A/C122A; PNGase, peptide-N-glycosidase; SEE, staphylococcal enterotoxin E; WT, wild type; DSS, disuccinimidyl suberate.
glycosylation sites were mutated. Interestingly, this triple-mutant N78A/N110A/C122A (NNC) CTLA-4 was still functional, localized in the IS, and inhibited T cell activation. Such functionality correlated with NNC CTLA-4 becoming predominantly dimeric upon ligation with B7. Our findings demonstrate that CTLA-4 dimerization/oligomerization is hierarchically regulated by intermolecular disulfide bonding, N-linked glycosylation, and B7 ligand-driven dimerization. This provides a structural framework for CTLA-4 lattice formation leading to B7-dependent functions of CTLA-4.

Materials and Methods

Plasmids and T cell transfectants

CTLA-4 mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene) on the previously described doxycycline-inducible plpBIG2i vector containing wild-type (WT) CTLA-4 as a template (19, 20). The N→A mutations were made on pBluescript vector containing WT CTLA-4 or C122A CTLA-4 and subcloned into plpBIG2i using the Gateway kit with pENTR/d/stopo as a shuttle vector (Invitrogen Life Technologies). Stable transfectants were generated by electroporating linearized plasmids into Jurkat E6.1 T cells and were screened for stable expression as described previously (20). Individual clones with high levels of CD3, CD28 and doxycycline-inducible expression of CTLA-4 were selected for study. Jurkat T cells and B lymphoblast cell lines were cultured under standard conditions in R-10 medium (21). Doxycycline (Sigma-Aldrich) was added in culture at night over 100 ng/ml for inhibition assays or 1000 ng/ml for biochemical experiments (8).

Antibodies

Mouse anti-human CTLA-4 mAbs 11 and 24 were supplied by Dr. B. M. Carreno (Wyeth Research, Cambridge, MA) and used for immunoblotting and immunoprecipitation respectively. For flow cytometry and confocal microscopy, FITC-conjugated anti-human IgG, Fcγ fragment-specific Ab (Jackson Immunoresearch Laboratories) and FITC-labeled anti-CD3 (UCHT-1), FITC-labeled anti-CD28, and PE-labeled anti-CTLA-4 (BNI3) mAbs (BD Pharmingen) were used. Immunoblotting for ERK-1/2 was performed using a rabbit polyclonal immunooaffinity-purified antisera (Stressgen Biotechnologies). Linker for activation of T cells (LAT) was immunoblotted against nuclear transfer (Santa Cruz Biotechnology). Actin was immunoblotted using an anti-LAT rabbit polyclonal antisera (Upstate Biotechnology). Ezrin was immunoblotted with a goat polyclonal antiserum against ezrin (Santa Cruz Biotechnology). Actin was immunoblotted using an affinity-purified goat polyclonal antisera (Santa Cruz Biotechnology). Isotype control immunoprecipitations were performed with a mouse IgG1 (eBioscience). Immunoprecipitation of CTLA-4 with B7 was performed with B7.2-lg (Genetics Institute/Wyeth Research).

Confocal microscopy

For confocal microscopic analysis of IS formation, T cells (1.0 × 10^6 cells/group) were stimulated with LG2 B lymphoblastoid (1.0 × 10^6 cells/group) and staphylococcal enterotoxin E (SEE; 100 ng/ml; Toxin Technology) on poly-L-lysine-coated confocal dishes (MatTek) as described previously (8). Cells were first fixed with 4% paraformaldehyde, then stained with PE-labeled anti-CTLA-4 mAb and FITC-labeled anti-CD3 mAb for 30 min on ice. Samples were analyzed on a confocal microscope (Zeiss) and LSM 510 software (Zeiss).

T cell functional assays

T cells (0.2 × 10^6 cells/group), with or without preincubation with doxycycline (100 ng/ml) to induce CTLA-4 expression, were plated in triplicate on 96-well plates with the B lymphoblast LG2 (0.1 × 10^6 cells/group) with or without 10 ng/ml SEE or with or without 0.1 ng/ml SEE in the presence or the absence of 100 ng/ml doxycycline at 37°C for 48 h. Supernatants were collected and diluted 1/10 before measurement of IL-2 by ELISA.

Flow cytometry

T cells (1.0 × 10^6 cells/group) were washed twice with PBS, resuspended, and stained with FITC-labeled anti-CD3, FITC-labeled anti-CD28, or PE-labeled anti-CD3 mAb for 30 min on ice. Cells were washed twice with PBS and kept on ice during the procedure. Flow cytometry was performed on a FACScalibur, and analysis was conducted using CellQuest software (BD Biosciences).

Cell surface biotinylation

Stable T cell transfectants (40 × 10^6 cells/group) were induced overnight for CTLA-4 expression with doxycycline (5 µg/ml), washed four times with PBS, treated with 0.5 mg/ml sulfosuccinimidyl-6-(biotinamido)hexanoate solution (20 mg/ml in PBS, pH 8.0; Pierce), and rotated slowly end over end for 30 min at room temperature to prevent precipitation of the normal human serum-biotin reagent. Cells were then washed three times with PBS at room temperature, and lysates were prepared and used for standard biotin and CTLA-4 immunoprecipitations. Dimerization of CTLA-4 under nonreducing conditions was tested in experiments in which the sample buffer did not contain 2-ME.

Raft isolation and biochemistry

Lipid rafts were isolated by sucrose gradient ultracentrifugation after lysis with 0.5% Triton X-100 as reported previously (8). Lipid rafts were pelleted by centrifugation of the 1-ml raft fraction for 1 h at 14,000 rpm 4°C and were resuspended in lysis buffer and sample buffer for biochemistry. For lysates and immunoprecipitations under nonreducing conditions, sample buffer without 2-ME was used. Chemiluminescence was detected using a digital image analyzer (Alpha Imotench). Densitometry and molecular mass calibration were performed with the Phoretix 1D Database package (Nonlinear Dynamics).

Tunicamycin, brefeldin A, peptide: N-glycosidase-F (PNGase-F), N-ethylmaleimide, and disuccinimidyl suberate (DSS) treatments

T cells (5 × 10^6 cells/group) were preincubated with brefeldin A or tunicamycin (Sigma-Aldrich) at 1 µg/ml for 1.5 h. Then 5 µg/ml doxycycline were added for 8 h more in the presence of these drugs. Cells were washed in PBS and lysed, and the whole cell lysates were used for immunoblotting under nonreducing or reducing conditions. PNGase-F treatment was performed according to the manufacturer’s specifications (New England BioLabs). Briefly, lysates from T cell transfectants induced to express CTLA-4 with 5 µg/ml doxycycline for 18 h were boiled at 100°C for 10 min in a SDS- and 2-ME-containing buffer. Lysates were then incubated at 37°C with 1% Nonidet P-40 and 200 U of PNGase F for 1 h. For N-ethylmaleimide treatment (Sigma-Aldrich), this reagent, at a final concentration of 10 mM, was added to lysates before boiling, allowed by addition of nonreducing sample buffer, boiling for 10 min, SDS-PAGE, and immunoblotting. For DSS treatment, a stock 25 mM DSS (Pierce) in DMSO or DMSO.

FIGURE 1. In vivo CTLA-4 dimerization in the absence of intermolecular disulfide bonding at C122. A, Dimerization of WT CTLA-4 and C122A CTLA-4 was determined by immunoblotting whole cell lysates (1.0 × 10^6 cell equivalents/sample) from stable Jurkat T cell transfectants for WT or C122A CTLA-4 under nonreducing (left panel) or reducing (right panel) conditions. B, The proportion of CTLA-4 dimers in nine independent WT CTLA-4-transfected T cell clones (average, 89%; SD, 13%) and in nine independent C122A CTLA-4-transfected T cells (average, 36%; SD, 10%) was calculated by band densitometry. The proportion of CTLA-4 dimers was calculated by taking the densitometric reading of the reduction-sensitive band (dimeric CTLA-4) under nonreducing conditions divided by the total densitometric reading for the reduction-sensitive and -insensitive bands (dimeric CTLA-4 and monomeric CTLA-4). For quantification of C122A monomers, the two reduction-insensitive bands were considered the monomer fraction. *** p < 0.001.
FIGURE 2. C122A CTLA-4 compartmentalizes in lipid rafts, is expressed on the T cell surface, and relocates to the IS upon coligation with the TCR.

A. The lipid raft fraction and the soluble fraction (which contains cytosol and nonraft membranes) from 4 × 10⁶ stable Jurkat T cell transfectants expressing WT CTLA-4 or C122A CTLA-4 were purified using sucrose gradient centrifugation after lysis in 0.5% Triton X-containing buffer. The fraction containing lipid rafts was pelleted and loaded, whereas the soluble fraction represents 3.0 × 10⁵ cell equivalents. Membranes were probed for CTLA-4 under nonreducing conditions. The mobility of the soluble fraction is slower due to the presence of a high concentration of sucrose in the sample. Membranes were stripped and reblotted for ERK-1/2 as a control for the soluble fractions and for LAT as a control for the raft fractions.

B. Surface expression of CD3, CD28, and WT CTLA-4 or C122A CTLA-4 was determined by flow cytometry in the presence (solid line) or the absence (dotted line) of doxycycline. The gray line is the negative control.

C. The dimerization status of the surface pool of WT CTLA-4 or C122A CTLA-4 was determined by cell surface biotinylation of doxycycline-induced WT and C122A CTLA-4 T cell transfectants (4 × 10⁶ cells/group). (Figure legend continues)
alone (as a control) was added to T cells to a final concentration of 2 mM DSS. Samples were rotated for 30 min at room temperature or for 2 h on ice, then quenched with 20 mM Tris for 15 min at room temperature, washed twice with PBS, lysed, and immunoblotted under reducing conditions.

**Results**

**CTLA-4 dimerizes in vivo in the absence of intermolecular disulfide bonding at C122**

A previous report had indicated that C122 is required for dimerization of soluble CTLA-4 (15). To study the functional implications of CTLA-4 dimerization in T lymphocytes, we introduced a cysteine to alanine point mutation in that position (C122A), subcloned the mutant CTLA-4 cDNA into a doxycycline-inducible plasmid, and generated stably transfected Jurkat T cells. These cells are an appropriate cell line for structural analysis of CTLA-4 because they do not express endogenous CTLA-4 even upon activation (22), and they preserve a glycosylation machinery comparable to that of peripheral blood T lymphocytes (23). Disruption of intermolecular disulfide bonds by mutating cysteine residues to alanine residues has been validated in a variety of dimeric or oligomeric protein complexes (24–26). Interestingly, we found that C122A CTLA-4 could still dimerize in significant amounts, as illustrated by the presence of a mixture of monomers and reduction-sensitive dimers in Western blots under nonreducing conditions (Fig. 1A). Quantification of monomeric and dimeric forms from multiple stably transfected T cell clones indicated that, on the average, 35% of C122A CTLA-4 were dimeric, although the percentage of dimeric C122A CTLA-4 was as high as 45%, compared with 90% or more for WT CTLA-4 (Fig. 1B). We also noticed a difference in the gel mobility of C122A CTLA-4 that was due to aberrant N-linked glycosylation of this mutant form of CTLA-4 (see below). The observed dimerization of C122A CTLA-4 was not due to intragel dimerization, because it was still present when the T cell lysates were prepared in the presence of excess N-ethylmaleimide, a reagent that chemically modifies free cysteine residues and thus prevents de novo formation of disulfide bonds (data not shown). Moreover, C122A CTLA-4 dimers were preserved under reducing conditions if DSS, a membrane-permeable, 11.4-Å arm length cross-linker, was added before lysing the cells, indicating that C122A CTLA-4 dimers are present in intact cells (data not shown). We also noted the presence of a lower band in the C122A CTLA-4 lysates under reducing and nonreducing conditions representing nonglycosylated and/or partially glycosylated CTLA-4.

**CTLA-4 is still functional in the absence of intermolecular disulfide bonding at C122**

Previous reports have demonstrated that CTLA-4 is in lipid rafts under conditions of T cell inactivation (8, 9). To characterize the biological properties of C122A CTLA-4, we first determined whether this mutant form of CTLA-4 partitioned in lipid rafts. As shown in Fig. 2A, similar amounts of WT and C122A CTLA-4 localized within lipid rafts, indicating that C122A CTLA-4 is poised to inhibit T cell activation. The quality of the raft fractionation procedure was confirmed by blotting for ERK-1/2 as a soluble fraction control and for LAT as a raft fraction control. Next, we examined whether C122A CTLA-4 reached the T cell surface, because the surface pool is the one that interacts with B7 ligands. We found that C122A CTLA-4 was expressed on the T cell surface (Fig. 2B). To determine the dimerization status of the T cell surface pool of C122A CTLA-4, we analyzed these transfectants after cell surface biotinylation. We found that upon immunoprecipitation surface CTLA-4 molecules, the pool of C122A CTLA-4 on the cell surface was enriched for dimeric forms (Fig. 2C). The 2- to 3-fold enrichment of C122A CTLA-4 dimers on the cell surface suggests a preferential export pathway for CTLA-4 dimers that is currently under investigation. Another key feature of CTLA-4 function is its partitioning within the IS under conditions of T cell inactivation (8, 9, 11). Thus, we examined whether C122A CTLA-4 moved to the IS upon coligation with the TCR. As shown in Fig. 2D, C122A CTLA-4 relocated to the IS when coligated with the TCR under conditions of SEE and APC stimulation. In this system, we have previously shown that WT CTLA-4 undergoes similar relocation (Fig. 2D) (8). Quantification of CD3-CTLA-4 cocapping under conditions of SEE-induced IS formation indicated that WT CTLA-4 and C122A CTLA-4 are able to localize to the IS to a similar degree (Fig. 2D). Taken together, these results indicate that the subcellular and molecular distributions of C122A CTLA-4 are comparable to those of WT CTLA-4.

Because C122A CTLA-4 was distributed in a similar fashion as WT CTLA-4, we assessed the ability of this mutant CTLA-4 to inhibit SEE-induced T cell activation. We found that the expression of C122A CTLA-4 on T cells caused a significant inhibition of IL-2 production in response to SEE stimulation (Fig. 3). Although the degree of IL-2 inhibition observed in these assays is relatively minor (40–60%), it is consistently detected at the levels of CTLA-4 expressed in these experiments, and these levels are comparable to those established previously (6, 8, 27). Thus, despite the absence of intermolecular disulfide bonding at C122, C122A CTLA-4 can still be expressed in dimeric form on the T cell surface, partition within lipid rafts, colocalize with the TCR in the IS, and inhibit T cell activation. These results demonstrate that biochemical forces other than the intermolecular disulfide bonding at C122 contribute to CTLA-4 dimerization and function.

**N-glycosylation is required for CTLA-4 dimer stabilization in the absence of C122**

As indicated above, we observed that C122A CTLA-4 monomers and dimers had a slower mobility than WT CTLA-4 in SDS-PAGE (Fig. 1). One possibility to explain the different mobility of C122A CTLA-4 is aberrant glycosylation. CTLA-4 has not been reported to have O-linked glycosylation, and we found that enzymatic deglycosylation with a mixture of enzymes to remove O-glycans had no significant effect on the mobility of CTLA-4 (data not shown). In contrast, CTLA-4 is N-glycosylated, with two known N-linked glycosylation sites at residues N78 and N110. These residues are followed by cell lysis, immunoprecipitation with anti-biotin or anti-CTLA-4 Abs, and CTLA-4 immunoblotting under nonreducing conditions. The immunoprecipitation beads treated with lysis buffer alone are shown as controls. The exposure of the biotin immunoprecipitation membrane was increased to facilitate qualitative comparison with the CTLA-4 immunoprecipitation membrane. D, WT, C122A, and NNC CTLA-4-expressing stable T cell transfectants were stimulated with APC and SEE (as indicated in Materials and Methods) and analyzed by confocal microscopy. CTLA-4 and CD3 were visualized with Abs labeled with PE (red) and FITC (green), respectively. The brightfield image is shown in the top right quadrant, and an overlay of red-green and light channel channels is shown in the bottom right quadrant, where a yellow signal indicates red-green overlap. White arrows indicate the location of putative IS. Synapse formation was quantified by counting at least 50 T cell/APC doublets per group. Cocapping of CD3 and CTLA-4 was scored when both colocalized at the interface between the T cell and the APC. The percentage of doublets was determined by dividing the number of CD3:CTLA-4 cocaps by the total number of doublets counted. Cocapping was 88% for WT CTLA-4, 90% for C122A CTLA-4, and 85% for NNC CTLA-4.
located in its extracellular domain (28, 29). To test whether the lower mobility of C122A CTLA-4 in SDS-PAGE was due to N-glycosylation, we treated the whole cell lysates from the WT CTLA-4 and C122A CTLA-4 stable Jurkat T cell transfectants with PNGase-F, which cleaves off N-linked glycans, and immunoblotted these lysates for CTLA-4. We observed that after PNGase treatment, a single band of identical mobility for both WT CTLA-4 and C122A CTLA-4 was detected (Fig. 4A). A similar result was obtained upon incubation of the WT CTLA-4 and C122A CTLA-4 transfectants with tunicamycin before lysis, a technique that prevents the addition of N-linked glycans during biosynthesis (data not shown). Therefore, the lower mobility of C122A CTLA-4 in SDS-PAGE correlates with aberrant N-glycosylation.

To test whether N-glycosylation contributed to CTLA-4 dimerization in the absence of C122, we incubated WT CTLA-4 and C122A CTLA-4 T cell transfectants with brefeldin A, a fungal metabolite that impairs the transport to the Golgi apparatus and causes retention of proteins in the endoplasmic reticulum (ER) (30). We observed that treatment with brefeldin A resulted in a single band identical for WT CTLA-4 and C122A CTLA-4 under reducing conditions (Fig. 4B, left panel). Interestingly, when the same samples were run under nonreducing conditions, the amount of dimeric C122A CTLA-4 was significantly diminished upon treatment with brefeldin A, whereas the amount of WT CTLA-4 dimers remained unaffected (Fig. 4B, right panel). Taken together, these data indicate that in the absence of intermolecular disulfide bonding at C122, CTLA-4 undergoes aberrant N-glycosylation in the Golgi, and this unique glycosylation contributes to stabilizing C122A CTLA-4 dimers.

To further test the role of N-glycosylation on CTLA-4 oligomerization, we generated a panel of point mutations at the N110 and/or N78 glycosylation sites of the WT CTLA-4 or C122A CTLA-4 and assessed the formation of dimers of these mutant CTLA-4 molecules. As shown in Fig. 5A, single N110A or N78A mutations or the N78A/N110A double mutation on the WT CTLA-4 background did not prevent dimerization. Note that the mobility of the dimers changed accordingly to the loss of N-glycosylation. On the C112A CTLA-4 background, N110A point mutation alone did not affect the proportion of dimers. However, N78A/N110A double mutation on the C122A background (triple mutant; NNC CTLA-4) resulted in monomeric CTLA-4 (Fig. 5A). Upon reduction, the intensity of the NNC CTLA-4 bands reproducibly decreased, implying that a reduction-sensitive mechanism contributed to the stabilization of these molecules. These data lead us to conclude that N-glycosylation of CTLA-4 molecules contributes to the formation of CTLA-4 dimers, an effect that can overcome the lack of C122-dependent covalent bonding. It is important to note that the loss of dimerization for the NNC CTLA-4 mutant is not due to misfolding secondary to the lack of glycosylation, because the N78A/N110A mutations on the WT CTLA-4 background did not prevent dimerization. Molecular mass calibration on multiple independent experiments with several different clones revealed that on the WT background, glycosylation on N110 and N78 account for 6 and 3 kDa of the molecular mass of human CTLA-4, respectively, whereas on the C122A background, these numbers increase to 8.5 and 5, respectively, whereas the CTLA-4 polypeptide accounts for 15–20 kDa under reducing conditions (Fig. 5B).

**Monomeric CTLA-4 functions normally and undergoes B7 ligand-dependent dimerization**

Because NNC CTLA-4 failed to dimerize, we wanted to further characterize its ability to fold properly and inhibit T cell activation. First, we examined whether the NNC CTLA-4 was expressed on the T cell surface. We found that the glycosylation mutants on the...
WT CTLA-4 and C122A CTLA-4 background were expressed on the T cell surface, as determined by flow cytometry. In particular, the NNC CTLA-4, which was monomeric, was expressed on the cell surface, as determined by flow cytometry (Fig. 6A). Also, this mutant form of CTLA-4 colocalized with the TCR in the IS upon coligation with the TCR, as did WT CTLA-4 (Fig. 2D). More importantly, as shown in Fig. 6B, NNC CTLA-4 significantly inhibited IL-2 production by T cells in response to SEE. The ability of NNC CTLA-4 to inhibit IL-2 production was comparable to that of WT CTLA-4 upon normalization of absolute IL-2 production under conditions of maximal CTLA-4 induction with doxycycline. These results indicate that NNC CTLA-4 folds appropriately, because proper folding is necessary to maintain the conformation of the B7 binding motif (28). If NNC CTLA-4 or C122A CTLA-4 were misfolded, the B7 binding motif would probably be perturbed, thus preventing CTLA-4-mediated T cell inactivation.

To explain the mechanistic basis for the function of NNC CTLA-4, we hypothesized that the presence of a bivalent CTLA-4 ligand on the surface of an APC could induce dimerization of NNC CTLA-4. To test this hypothesis, whole cell lysates from stable WT CTLA-4, C122A CTLA-4, and NNC CTLA-4 T cell transfectants were immunoprecipitated with recombinant B7.2-Ig and analyzed for dimer formation under nonreducing conditions. WT CTLA-4, C122A CTLA-4, and NNC CTLA-4 were able to bind to B7.2-Ig to a similar extent on the cell surface (Fig. 7A). We found that B7.2-Ig pulldown caused a significant increase in the proportion of C122A and NNC dimers compared with that seen in whole cell lysates (Fig. 7B). This was particularly apparent for the NNC CTLA-4, for which dimers were not detected in whole cell lysates, but constituted the majority of the CTLA-4 signal in B7.2-Ig pull-down experiments. The C122A CTLA-4 transfectant selected for this experiment expressed a lesser amount of dimer than the average C122A CTLA-4 transfectant to better illustrate the B7.2-Ig-induced dimerization. Therefore, B7 ligands can drive dimerization of monomeric CTLA-4 on the cell surface, and this is associated with normal function of monomeric CTLA-4.
addition, as shown in this study, CTLA-4 dimers are rescued by aberrant N-glycosylation in T cells, which may not be operational or may be different in COS cells. The second key finding of our studies is that N-glycosylation of CTLA-4 plays a key role in facilitating CTLA-4 dimerization, particularly in the absence of C122-dependent covalent bonding. If it is N-glycosylated, then the mutant C122A CTLA-4 can still partition within lipid rafts, is expressed on the T cell surface, colocalizes with the TCR in the IS, and is fully functional. Our data with PNGase-F and brefeldin A indicate that the rescuing of C122A CTLA-4 by N-glycosylation occurs in the N-glycan maturation process within the Golgi as opposed to the initial addition of immature N-glycans in the ER. It is important to note that the lack of C122 and N-glycosylation sites did not lead to misfolding based on the evidence that the NNC mutant CTLA-4 molecules trafficked to the cell surface, were still recognized by anti-CTLA-4 Abs, relocated to the IS, were able to bind B7, and inhibited T cell activation. These findings would not be possible if CTLA-4 was grossly misfolded.

The relationship between glycosylation, receptor dimerization, and receptor function has been examined in a few studies, although no single paradigm has emerged. Glycosylation can either enhance or interfere with receptor dimerization. For example, glycosylation at residue N15 is required for β1-adrenergic receptor dimerization and surface expression (31), and deglycosylation of the oncogenic truncated epidermal growth factor receptor impairs its homodimerization and kinase activity (32). In addition, single N-linked glycosylation has been shown to contribute to disulfide-dependent dimer formation of mucins (33). In contrast, glycosylation (sialylation and O-glycosylation) negatively modulates CD45 dimerization (34–37). Nevertheless, in some cases, glycosylation has no effect on dimerization, as seen with CD26, a serine protease with nine glycosylation sites that retains its ability to dimerize and function in the absence of these N residues (38). Differential glycosylation also has functional consequences, some of them through its effects on receptor dimerization. For example, on CD45, glycosylation decreases dimerization, and this is associated with increased activity of this phosphatase (34–37). In this sense, it is of interest to point out that T cells express a hypoglycosylated form of CD86 activity of this phosphatase (34–37). In this sense, it is of interest to point out that T cells express a hypoglycosylated form of CD86 with reduced binding affinity for CTLA-4 and CD28 and thus may act as a regulator of T cell activation (39). N-linked glycosylation of CD28 can also negatively regulate its binding to CD80 and thus inhibit its costimulatory function.

There are several possible mechanisms to account for the contribution of N-glycosylation to CTLA-4 dimerization. The crystal structure of B7.1-engaged CTLA-4 showed that the N-linked oligosaccharide at residue N110 is oriented toward the B7.1-ligand and is thought to hide a hydrophobic patch that can lead to aggregation of CTLA-4 when the N78 residue is mutated (28). Another possibility is that N-glycosylation stabilizes the small noncovalent binding surface at the dimerization interface. The third key finding of our studies is that the NNC mutant CTLA-4 molecules trafficked to the cell surface, were still recognized by anti-CTLA-4 Abs, relocated to the IS, and is fully functional. Our data with PNGase-F and brefeldin A indicate that the rescuing of C122A CTLA-4 by N-glycosylation occurs in the N-glycan maturation process within the Golgi as opposed to the initial addition of immature N-glycans in the ER. It is important to note that the lack of C122 and N-glycosylation sites did not lead to misfolding based on the evidence that the NNC mutant CTLA-4 molecules trafficked to the cell surface, were still recognized by anti-CTLA-4 Abs, relocated to the IS, and is fully functional. Our data with PNGase-F and brefeldin A indicate that the rescuing of C122A CTLA-4 by N-glycosylation occurs in the N-glycan maturation process within the Golgi as opposed to the initial addition of immature N-glycans in the ER. It is important to note that the lack of C122 and N-glycosylation sites did not lead to misfolding based on the evidence that the NNC mutant CTLA-4 molecules trafficked to the cell surface, were still recognized by anti-CTLA-4 Abs, relocated to the IS, were able to bind B7, and inhibited T cell activation. These findings would not be possible if CTLA-4 was grossly misfolded.

FIGURE 7. Ligation with monomeric B7.2-Ig induces CTLA-4 dimerization. A, Binding of B7.2-Ig to WT CTLA-4, C122A CTLA-4, and NNC CTLA-4 stable Jurkat T cell transfectants was determined by incubating cells with B7.2-Ig, followed by staining with a FITC-labeled anti-human Fcγ-specific Ab and subsequent FACS. B, WT CTLA-4, C122A CTLA-4, and NNC CTLA-4 stable Jurkat T cell transfectants were lysed, CTLA-4 precipitated with B7.2-Ig on protein G beads, and immunoblotted for CTLA-4 under nonreducing conditions. C122A CTLA-4 and NNC CTLA-4, which were mostly monomeric in the whole cell lysates, were predominantly dimeric upon B7.2-Ig binding. Nontransfected Jurkat T cells (E6.1; right panel) and B7.2-coated beads treated with lysis buffer (first lane) are shown as negative controls. The image intensity of the lysate panel was increased to facilitate qualitative comparison with the immunoprecipitation panel.

Discussion
Recent crystallographic studies have suggested that CTLA-4-B7 lattices provide the mechanistic basis for triggering the inhibitory function of CTLA-4 (14, 16). To test the biological relevance of CTLA-4-B7 lattices in vivo, we generated a monomeric version of CTLA-4, reasoning that monomeric CTLA-4 would not be able to drive lattice formation with B7. To do this, we mutated residue C122 of CTLA-4 to alanine, because a previous report had shown that C122 forms an intermolecular disulfide bond that is essential for homodimerization in solution (15). These studies provide new insights on the regulation of CTLA-4 oligomerization.

The first remarkable finding is that, contrary to what was expected (15), CTLA-4 in T cells can still dimerize in the absence of C122, with up to 35% of C122A CTLA-4 being dimeric. Such a form can reach the T cell surface, is fully functional, and does not misfold based on its ability to be detected by anti-CTLA-4 Abs and by the fact that it binds B7. The apparent discrepancy between this finding and that of Linsley et al. (15) may be due to the fact that we used intact CTLA-4 expressed in T cells, whereas Linsley et al. used soluble CTLA-4 cleaved from the surface of COS cells. In addition, as shown in this study, CTLA-4 dimers are rescued by aberrant N-glycosylation in T cells, which may not be operational or may be different in COS cells. The second key finding of our studies is that N-glycosylation of CTLA-4 plays a key role in facilitating CTLA-4 dimerization, particularly in the absence of C122-dependent covalent bonding. If it is N-glycosylated, then the mutant C122A CTLA-4 can still partition within lipid rafts, is expressed on the T cell surface, colocalizes with the TCR in the IS, and is fully functional. Our data with PNGase-F and brefeldin A indicate that the rescuing of C122A CTLA-4 by N-glycosylation occurs in the N-glycan maturation process within the Golgi as opposed to the initial addition of immature N-glycans in the ER. It is important to note that the lack of C122 and N-glycosylation sites did not lead to misfolding based on the evidence that the NNC mutant CTLA-4 molecules trafficked to the cell surface, were still recognized by anti-CTLA-4 Abs, relocated to the IS, were able to bind B7, and inhibited T cell activation. These findings would not be possible if CTLA-4 was grossly misfolded.

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The sensitivity to reduction would imply that a disulfide bond is involved in this process. There are two intramolecular disulfide bonds per CTLA-4 monomer: one between C21 and C92, which is involved in forming the classical Ig fold and is required for maintaining the conformation of the B7 binding motif, and the second between C48 and C66, which is unique to CTLA-4 and has no known function (15). These intramolecular disulfide bonds may indirectly stabilize C122A dimers in a manner analogous to what has been reported for mucins, which can form intramolecular disulfide-dependent cysteine knots (33). Although no such cysteine knot was seen in WT CTLA-4 crystal structures, we cannot rule out such an effect in the C122A mutant. Another possibility is that the C48-C66 bond, which is found close to the dimerization interface (16), could form a novel intermolecular disulfide bond during biosynthesis. We ruled out the possibility of intragel dimerization through de novo disulfide bond formation by treating the cells with N-ethylmaleimide before lysis, which blocks exposed cysteine residues from bonding. Also, the NNC CTLA-4 mutant has all the other cysteine residues intact and still does not dimerize, thus making it unlikely that these other cysteine residues contribute to the formation of C122A dimers. The contribution of glycosylation in this respect could be to slow the rate of biosynthesis and facilitate interactions with chaperones in the ER, as was reported for mucin (33). However, we have shown that dimerization of C122A and NNC CTLA-4 could occur upon ligation with monomeric B7-Ig during immunoprecipitation, suggesting that mature molecules are also capable of forming stable dimers.

Based on the data presented in this study, we propose a hierarchical model of CTLA-4 oligomerization leading to B7:CTLA-4 lattice formation and the triggering of B7-dependent inhibitory functions of CTLA-4. In this model there are three separate steps that supersede each other in a sequential fashion. These are C122-dependent covalent bonding, N-glycosylation, and binding to B7. In the ER, disulfide bonds at C122 form and generate CTLA-4 dimers. In the event that this covalent bond cannot form, oligosaccharide modifications completed in the Golgi allow for dimerization of CTLA-4. Nevertheless, in the absence of N-glycosylation of the molecule (e.g., similar to what has been reported to occur in vivo for B7 (39)), monomeric CTLA-4 can reach the T cell surface, where it will dimerize after binding to B7. This allows for monomeric CTLA-4 to form lattices and inhibit T cell activation in a B7-dependent fashion. It remains to be determined whether the fine architecture of CTLA-4 oligomers induced by B7 in the presence or the absence of disulfide bond/N-glycosylation-dependent dimers is the same, and whether the redistribution of these oligomers changes during T cell activation (41).

Although spontaneous mutations of the C122 residue in CTLA-4 have not been reported in a clinical setting, conditions of cellular stress (e.g., oxidative stress) could cause defects in glycosylation and cysteine-based dimerization/oligomerization of CTLA-4 leading to the generation of monomeric CTLA-4. This possibility has been documented for other molecules. In the case of receptor-like protein phosphatase-α, oxidative stress leads to a conformational change in the cytoplasmic tail of the molecule that is subsequently transmitted to the extracellular portion of the molecule and modulates dimer formation and the function of the protein (42, 43). The tumor suppressor ARF also reversibly forms homo-oligomers under oxidizing conditions, and such oligomers can form even after the three sole cysteine residues in this protein are deleted (44). Disulfide bond-independent oligomerization has also been shown for the tumor suppressor B23, which forms oligomers under reducing conditions (45). Finally, the DNA-binding activity of NtcA was perturbed by the reducing agent DTT, and this effect was preserved in NtcA cysteine mutants (46). Thus, the results presented in this study have direct biological implications on CTLA-4 function. By analogy to these reported cases, it is plausible to conclude that the capacity of B7 binding to determine the formation of functional dimers of CTLA-4 is a safe mechanism to avoid loss of function of CTLA-4 under conditions of redox stress after T cell activation.

In closing, it is important to note that the model proposed in this study for CTLA-4 oligomerization is operational for those CTLA-4 functions that are B7 dependent. Recently, there has been increasing interest in B7-independent functions of CTLA-4 (47). The different biological implications for B7-dependent and B7-independent CTLA-4 functions and their differential structural requirements may have contributed to the selective advantage for such elaborate regulation of CTLA-4 dimerization/oligomerization.

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Disclosures
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