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Molecular Determinants of Inverse Agonist Activity of Biologicals Targeting CTLA-4

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Ligation of CD28 or CTLA-4 with some biologicals can activate T cells due to an unexpected superagonist or inverse agonist activity, respectively. The risk of such an outcome limits the therapeutic development of these reagents. Thus, identifying the molecular determinants of superagonist/inverse agonist properties for biologicals targeting costimulatory/inhibitory receptors has not only fundamental value but also important therapeutic implications. In this study, we show that ligation of CTLA-4 with either soluble B7.1 Ig (but not B7.2 Ig) or with a recombinant bispecific in-tandem single chain Fv known as 24:26 induces TCR-independent, T cell activation. Such an inverse agonist activity requires CD28 expression and high CTLA-4 expression and is not seen when CTLA-4 is ligated by membrane-bound B7.1 or B7.2. At the molecular level, the inverse agonist activity of B7.1 Ig or 24:26 correlates with their ability to induce the formation of unique dimer-based, CTLA-4 oligomers on the T cell surface and involves CTLA-4 signaling through its cytoplasmic domain. Our results provide a potential mechanism to explain and to predict inverse agonist activity for CTLA-4 ligands. The Journal of Immunology, 2007, 179: 3631–3637.

Receptors CD28 and CTLA-4 are members of a family of receptors, derived from an ancestral common gene, that respectively costimulate or inhibit T cells in the context of TCR-dependent signaling (1). Despite their opposite functions and under unique circumstances, ligation of CD28 or CTLA-4 with biologicals or with recombinant Ab fragments can, by itself, induce T cell activation in a TCR-independent manner. For CD28, this has been shown with a subset of Abs known as superagonist Abs (2–4). For CTLA-4, in contrast, historical data had claimed the ability of some Abs to have inverse agonist properties (5–8), but this has only recently been demonstrated using a bispecific, in-tandem single chain Fv (ScFv)3 fragment against CTLA-4 known as 24:26 (9).

The pharmacological targeting of CD28 and CTLA-4 as an immunomodulatory strategy to down-regulate autoimmune responses and allograft rejection has generated a lot of interest (10, 11). However, the risk of inducing massive T cell activation, as recently documented in a phase I clinical trial with the CD28 superagonist Ab TGN1412 (12), limits the pharmacological development of these reagents. It would therefore be useful to have the capacity to predict whether a given biological targeting CD28 or CTLA-4 has superagonist or inverse agonist properties. To address this need, we embarked in a study to identify molecular features associated with the inverse agonist properties of CTLA-4 ligands. In this study, we show that the bispecific ScFv 24:26 and, surprisingly, soluble B7.1 Ig act as inverse agonists of CTLA-4 and induce IL-2 production in the absence of concomitant ligation of the TCR. Such an inverse agonist activity is not seen for B7.2 Ig or when B7.1 and B7.2 molecules are membrane bound. Furthermore, the inverse agonist properties of these reagents require CD28 expression and high surface expression of CTLA-4 and correlate with the formation of a unique dimer-based, CTLA-4 oligomer that signals through the cytoplasmic tail of CTLA-4. Based on our findings, we propose that the ability of a CTLA-4-targeting biological to induce the formation of dimer-based oligomers correlates with its inverse agonist properties. Assessing such capacity may minimize the risk and cost of further therapeutic development.

Materials and Methods

Cells

Jurkat E6.1 cells were obtained from American Type Culture Collection. The doxycycline-inducible, CTLA-4 stably transfected Jurkat T cell panel used for these studies has been extensively described (9, 13–17). The experiments reported here were done with wild-type CTLA-4 and with Y165F CTLA-4, a fully functional molecule that is expressed at high levels on the cell surface or with the indicated mutants. The NNYC CTLA-4 mutant was generated using a QuickChange site-directed mutagenesis kit (Stratagene) by adding the Y→F mutation at residue 165 to the previously described NNC CTLA-4 mutant (15). The B lymphoblastoid cell line LG-2 was provided by Dr. E. Long (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS.

Abs and reagents

The mouse mAbs 11 and 24, the ScFv molecules 24, 26, and 24:26 against human CTLA-4, mouse anti-human CTLA-4–26 Fab’, and B7.2 Ig were obtained from Wyeth Research (9, 13–17). The following commercially available Abs were used in these studies: a mouse mAb against CD45 (Transduction Laboratories and BD Biosciences), a goat polyclonal anti-serum against actin (Santa Cruz Biotechnology), an anti-human CD45 F(ab’)2 (Caltag Laboratories), and an anti-human CD28 Ab (BD Biosciences). A recombinant human B7.1/Fc chimera (B7.1 Ig) was purchased from R&D Systems. PE-labeled anti-B7.2 and PE-labeled IgG1 were purchased from eBioscience. PE-labeled anti-B7.1 was purchased from BD Biosciences. Staphylococcal enterotoxin E (SEE) was purchased from Toxin Technology. Disuccinimidyl suberate (DSS) was purchased from Pierce.
FIGURE 1. CTLA-4 can function as an inhibitor and as an activator of T cells depending on the ligand that it engages. A, Y165F CTLA-4 Jurkat T cells were treated with or without doxycycline and further cultured with LG-2 cells in the presence or absence of SEE (0.1 ng/ml) for 48 h. B, Y165F CTLA-4 Jurkat T cells were stimulated with 24:26 or 24 ScFv and 26 ScFv for 48 h at 37°C. C, Doxycycline-induced Jurkat cells transfected with Y165F CTLA-4 (control CTLA-4), M10 CTLA-4 (lacking the 24 binding site), M11 CTLA-4 (lacking the 26 binding site), or M10/M11 CTLA-4 (lacking both 24 and 26 binding sites) were stimulated with 24:26 at the concentrations indicated for 48 h at 37°C. D, Jurkat cells stably transfected with M10, M11, or M10/M11 CTLA-4 mutants were treated with or without doxycycline and cultured with LG-2 cells in the presence or absence of SEE (1.0 ng/ml) as in A. IL-2 production from all experiments was measured from the harvested supernatants by ELISA. **, p < 0.01; ***, p < 0.001.

T cell functional assays

Doxycycline-induced Jurkat E6.1 T cell transfectants (0.1 × 10⁶/group) were cultured with or without 24:26 or a mixture of 24 ScFv and 26 ScFv or SEE and LG-2 cells at the concentrations indicated and plated in triplicate in 96-well plates for 72 or 48 h, respectively, at 37°C (9). IL-2 in supernatants was measured by ELISA (BD Biosciences).

Flow cytometry

LG-2 cells (1 × 10⁶/group) were washed and stained with PE-labeled anti-B7.1 or anti-B7.2 or isotype-matched control. Samples were then washed in PBS and analyzed by flow cytometry (FlowJo; Tree Star).

Cellular fractionation and Western blotting

Doxycycline-induced, CTLA-4-expressing Jurkat cells (2 × 10⁶/group) were stimulated with or without 24:26, B7.1 Ig, B7.2 Ig, anti-CTLA-4 mAb 24, anti-CTLA-4-26 F(ab')2, anti-CD45 F(ab')2, or LG-2 cells pretreated with or without SEE for the indicated times at 37°C. The cells were further treated with or without DSS (2 mM in DMSO) for 30 min at room temperature. Cells were washed and subsequently lysed in standard lysis buffer containing Triton X-100 (1%) at 4°C for 30 min. Cell lysates were collected and an equal volume of lysis buffer was added to the remaining pellet fraction. The pellet fractions were resuspended using a Vibra Cell sonicator (Sonics and Materials). Protein samples were prepared in reducing (containing 2-ME) sample buffer as previously described (15). Protein samples were resolved by SDS-PAGE and analyzed by Western blotting using a digital image analyzer (Alpha Innotech).

Statistics

Unpaired Student’s t tests were performed using GraphPad Prism software. A difference between groups was considered significant when p ≤ 0.05.

Results

CTLA-4 can function as an inhibitor and an activator of T cells

As previously reported, ligation of CTLA-4 with B7 molecules on the surface on an APC results in the inhibition of superantigen-induced T cell responses (9, 13–17). To corroborate this inhibitory function of CTLA-4, we have extensively used an experimental system based on the response of doxycycline-inducible, wild-type or mutant CTLA-4 stably transfected Jurkat T cells to SEE and APC. In the absence of doxycycline in the culture medium, these T cells mount a very robust IL-2 response to SEE/APC. When doxycycline is added to the medium, these T cells express significant amounts of CTLA-4 in the cell surface and, under these conditions, the amount of IL-2 induced in response to SEE/APC is significantly abrogated (Fig. 1A).

In marked contrast to the above-mentioned results and as we have previously shown in primary human T cells and T cell lines, ligation of CTLA-4 with a bispecific, in-tandem ScFv known as 24:26, by itself, activated T cells as illustrated by the dose-dependent production of IL-2 of CTLA-4-expressing Jurkat T cells (Fig. 1B). Such a response was not observed without CTLA-4 expression and increased as the surface expression of CTLA-4 increased, as shown with the fully functional Y165F CTLA-4 variant that is expressed at high levels on the cell surface (9). In addition, the 24:26-induced IL-2 response was not observed when using unlinked 24 and 26 ScFvs. Therefore, from a pharmacological point of view, 24:26 acts as an inverse agonist of CTLA-4 because it converts this receptor from an inhibitor to an activator of T cells.

The inverse agonist activity of 24:26 required the presence of both CTLA-4 epitopes recognized by the 24 ScFv and the 26 ScFv, M10 (⁶⁶⁸E⁶⁸⁰) and M11 (⁶⁵⁶⁶⁸⁷⁶⁸⁸) respectively, because the IL-2 production in response to 24:26 was abolished when the M10 or M11 or both epitopes were mutated (Fig. 1C). However, mutations at either or both of these epitopes did not abolish the inhibitory function of CTLA-4 in response to its coligation with the TCR (Fig. 1D) (9). Of interest, we found that the inhibitory capacity of CTLA-4 mutants involving the M10 epitope was enhanced over that for the M11 epitope mutant CTLA-4 (inhibition of 50–75% of the IL-2 response vs 30%, respectively). The molecular basis for such an effect on B7-mediated inhibitory function of CTLA-4 is under investigation.

The inverse agonist properties of 24:26 correlate with its ability to induce a unique CTLA-4 dimer-based oligomer

Because the inverse agonist activity of 24:26 required the expression of both CTLA-4 epitopes recognized by this biological, we hypothesized that such an activity would correlate with the formation of a CTLA-4 oligomer. To test this hypothesis, we examined the presence of this oligomer by looking at the changes in CTLA-4 distribution in soluble and insoluble fractions upon ligation with 24:26 (Fig. 2). Immunoblotting of whole-cell lysates from 24:26 stimulated CTLA-4-expressing Jurkat cells revealed a consistent decrease in the level of CTLA-4 compared with that in nonstimulated T cells (Fig. 2A). The reduction of CTLA-4 (~30%) from the whole-cell lysate fraction prompted us to examine its redistribution to the remaining insoluble pellet fraction. We found that CTLA-4 was present in the insoluble fraction of cells stimulated with 24:26 (Fig. 2A). This correlated with a redistribution of surface CTLA-4...
from the membrane to the cytoskeletal fraction (data not shown). Neither decreased levels of CTLA-4 from the soluble lysate fraction or redistribution to the pellet were detected when CTLA-4-expressing T cells were stimulated with anti-CTLA-4 26 F(ab’)

2,
indicating that any bivalent ligation of CTLA-4 cannot induce this oligomerization. In addition, such a unique oligomerization was not observed for CD45 when this receptor was ligated with anti-CD45 F(ab’)

2.

To directly document the oligomerization of CTLA-4 induced by 24:26, we used chemical cross-linkers. The addition of a cell-impermeable, chemical cross-linker of short arm length before cell lysis revealed the presence of CTLA-4 tetramers and higher order oligomers in the insoluble pellet fractions of 24:26-stimulated T cells (Fig. 2B). Altogether, this evidence indicates that the CTLA-4 inverse agonist 24:26 induces the formation of a unique CTLA-4 oligomer, whose properties are not simply due to bivalent ligation.

CTLA-4 is expressed as a homodimer that is stabilized by a disulfide bridge at the cysteine residue 122 and by dual N-glycosylation at positions 78 and 110 (17). Given the above-mentioned data, we hypothesized that the unique oligomer induced by the inverse agonist 24:26 would require expression of intact CTLA-4 homodimers. To test this hypothesis, we used T cells expressing a monomeric form of CTLA-4 (known as NNCY CTLA-4) that cannot dimerize but is expressed on the cell surface (Fig. 3A) (17). We observed that 24:26 did not induce the unique CTLA-4 oligomer when T cells expressed monomeric CTLA-4 (Fig. 3B).

To further confirm that the inverse agonist activity of 24:26 correlated with its capacity to induce the formation of a dimer-based CTLA-4 oligomer, we tested whether T cells expressing the monomeric NNCY CTLA-4 mounted an IL-2 response to 24:26. We found that, in contrast to dimeric CTLA-4-expressing T cells (control CTLA-4), 24:26 failed to induce an IL-2 response to 24:26. We observed that 24:26 did not induce the unique CTLA-4 oligomer when T cells expressed monomeric CTLA-4 (Fig. 3B).

Soluble B7.1 Ig, but not membrane-bound B7 molecules, can act as an inverse agonist of CTLA-4

If the inverse agonist activity of a CTLA-4 ligand correlates with its ability to form a high-order CTLA-4 oligomer, then one should...
be able to generate inverse agonists of CTLA-4 by using recombinant versions of its natural ligands that force such an oligomerization. Two natural ligands of CTLA-4 have been reported to date: B7.1 and B7.2 (18–20). They have distinct profiles of expression and biochemical features, most importantly B7.1 has a higher affinity and avidity for CTLA-4 given its dimeric structure (21–23). Crystallographic data have suggested that ligation of CTLA-4 with B7.1, itself being a homodimer, may induce a lattice-like oligomer (17, 23, 24). With this in mind, we tested whether a soluble B7.1 and B7.2 (B7.1 Ig, B7.2 Ig) had inverse agonist properties on T cells expressing high levels of CTLA-4 on their cell surface.

We examined the ability of B7 molecules to induce the formation of a high-order CTLA-4 oligomer similar to that induced by 24:26. We found that engagement of CTLA-4 with soluble B7.1 Ig resulted in the formation of such an oligomer as detected by redistribution of CTLA-4 to the insoluble fraction (Fig. 4A). In contrast, soluble B7.2 Ig was not able to induce the formation of such an oligomer (Fig. 4A). Membrane-bound B7.1 and B7.2 molecules, expressed on LG-2 cells, as shown in Fig. 4B, were not able to induce such an oligomer either in the presence or absence of supernatant stimulation (Fig. 4A).

Based on the ability to induce high-order CTLA-4 oligomerization, we predicted that soluble B7.1 Ig but not B7.2 Ig or membrane-bound B7 molecules would have inverse agonist properties. To corroborate this prediction, we determined the capacity of these molecules to induce T cell activation through CTLA-4. To do this, we cultured Jurkat T cells with or without doxycycline to induce CTLA-4 expression and then stimulated these cells with 24:26 soluble B7.1 Ig and B7.2 Ig or membrane-bound B7 molecules (on LG-2 cells) for 48 h. As shown in Fig. 4C, IL-2 production was detected upon stimulation with 24:26 and with B7.1 Ig only under conditions of a high level of expression of CTLA-4, while B7.2 Ig and membrane-bound B7 molecules were unable to induce any IL-2 response. The lack of any significant IL-2 response by these T cells without induction of CTLA-4 expression indicated that 24:26 and B7.1 Ig act as inverse agonists of CTLA-4 and not as superagonists of CD28, which is expressed at high levels by these cells. The magnitude of IL-2 production induced by these biologicals likely reflects the differential affinities of these ligands for CTLA-4, being higher for 24:26 than for soluble B7.1 Ig, and this higher than for B7.2 Ig. Altogether, these data show that soluble ligands for CTLA-4 can act as inverse agonists of this receptor and induce T cell activation through an oligomerization-dependent manner.

The inverse agonist activity of biologicals targeting CTLA-4 requires the tail of CTLA-4

The inverse agonist response of these biologicals could primarily result from signaling through CTLA-4 or, alternatively, from signaling through another receptor trapped in these oligomers. To distinguish between these two options, we made use of Jurkat T cells expressing doxycycline-inducible CTLA-4 lacking the entire cytoplasmic tail (TLESS CTLA-4). First, we tested whether this biological would induce the unique CTLA-4 oligomer associated with inverse agonist activity. As one would expect based on the preservation of the extracellular domain of the tailless CTLA-4 molecule, we found that TLESS CTLA-4 still oligomerized in response to 24:26, as illustrated by the reduction of TLESS CTLA-4 from the whole-cell lysate fraction of 24:26-stimulated cells and subsequent appearance in the corresponding insoluble fraction (Fig. 5A). Thus, the oligomerization of CTLA-4 induced by 24:26 is not mediated by interactions through its intracellular domain.
Next, we tested whether the CTLA-4 oligomer signaled through the cytoplasmic domain of this receptor. This was done by stimulating TLESS CTLA-4-expressing T cells with 24:26 and measuring the IL-2 response. We found that ligation of TLESS CTLA-4 with 24:26 did not induce any IL-2 production (Fig. 5B). Therefore, we concluded that the inverse agonist activity of 24:26 results from signals relayed through the cytoplasmic tail of CTLA-4 oligomers.

The inhibitory and activating functions of CTLA-4 require CD28 expression

Microarray analysis of genes regulated by CTLA-4 ligation suggests that this receptor inactivates T cells by inhibiting CD28-dependent gene transcription (25). Based on this observation, we hypothesized that, by analogy to its inhibitory function, the inverse agonist response upon ligation of CTLA-4 involved cross-talk between signaling from this receptor and CD28 signaling. Thus, we tested whether the agonist and inverse agonist responses through CTLA-4 required concomitant expression of CD28 by using CD28+ and CD28− variants of CTLA-4-expressing T cells. We found that IL-2 production was inhibited by CTLA-4 in CD28+ T cells. However, we observed that ligation of CTLA-4 in CD28− T cells did not inhibit the IL-2 response to SEE:APC (Fig. 6A) despite the much reduced level of T cell activation of cells due to the lack of CD28 costimulation. The requirement for CD28 on CTLA-4-mediated inhibition of T cell activation was also applicable to the inverse agonist response through CTLA-4. As shown in Fig. 6B, the inverse agonist activity of 24:26 was only observed in T cells expressing high levels of CTLA-4 and CD28. Based on these data, we concluded that CD28 expression is required for the function of CTLA-4 both as an inhibitor and as an activator of T cells.

Discussion

The inhibitory function of CTLA-4 on T cell responses is well established in vitro and in vivo (1). However, early experimental data generated in vivo using Abs against CTLA-4 had suggested that, under certain conditions, this receptor can enhance T cell responses (5, 6). The complexity of this system made it difficult to interpret this result. More recently, though, we have been able to demonstrate that CTLA-4 can also function as an activator of T cells. This occurred when CTLA-4, expressed at high levels on the plasma membrane, was ligated with a recombinant, bispecific, Ab fragment called 24:26 (9). The mechanism for this unexpected inverse agonist activity was unknown. In this study, we show that the inverse agonist activity of not only 24:26 but also other soluble CTLA-4 ligands and biologicals correlates with their ability to induce a unique oligomerization state that relays activating signals through the cytoplasmic tail of CTLA-4. This ligand-induced architecture likely provides stability for the interaction of CTLA-4 with key signaling molecules that result in activating signaling and ultimately T cell activation.

The inverse agonist activity through CTLA-4 is not restricted to 24:26. We show here that soluble B7.1 Ig, but not B7.2 Ig, can act as an inverse agonist of CTLA-4. B7 molecules expressed on the surface of an APC are also unable to act as inverse agonists, suggesting a differential ability of soluble and membrane-bound B7.1 and B7.2 to engage CTLA-4, which may correlate with different binding affinities and avidity and different functional outcomes in vivo (1). The architectural constraint of molecules in a lipid bilayer may also provide restrictions in their ability to engage other receptors. Interestingly, a soluble form of B7.1 and B7.2 has been observed (26, 27) and elevated levels of soluble B7.1 and B7.2 are detected in patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (28–30). Of interest, although we have detected the inverse agonist activity of 24:26 in primary T cells consistently, the inverse agonist activity of B7.1 Ig may be dependent on the ability of primary T cells to up-regulate CTLA-4 at a very high density, a scenario that may occur only in a local microenvironment. Therefore, soluble B7.1 may act in vivo as an inverse agonist of CTLA-4, leading to the activation of potentially autoreactive T cells.

It is important to note that the redistribution of CTLA-4 to the insoluble fraction only represents the ability of a biological to induce the formation of a unique CTLA-4 oligomer, but it is not a quantitative indicator of the ability of that biological to induce T cell activation. As stated above, the magnitude of IL-2 production likely reflects the differential affinities of the ligands for CTLA-4. Because 24:26 is composed of scFv fragments of two specific Abs against CTLA-4, it likely has a higher affinity for CTLA-4 than soluble B7.1 Ig. The higher affinity may translate into a longer interaction with CTLA-4 allowing for sustained signaling and therefore higher levels of IL-2 production.

As implied by the differential effect of soluble B7.1 Ig vs membrane-bound B7.1, the structure of the activating CTLA-4 oligomer is distinctly different from the lattice-like oligomerization that is necessary for CTLA-4-mediated inhibition (17, 23, 24). The inhibitory lattice-like oligomer is regulated in a hierarchical manner by disulfide bonding, N-glycosylation, and B7 engagement and does not require homodimeric CTLA-4 (17). In addition, it can occur under conditions of low levels of expression of CTLA-4. In contrast, the activating oligomer requires the expression of intact CTLA-4 homodimers because monomeric CTLA-4 does not oligomerize under these conditions. Moreover, the inverse agonist activity of CTLA-4 requires high levels of surface expression of this receptor. Based on these observations, we hypothesize that the
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The biological significance of the data presented here is twofold. On one hand, it reconciles the historical observations of an activating role for CTLA-4 with the well-established inhibitory function of this receptor by providing a molecular explanation for both inhibitory and activating responses through CTLA-4 ligation. On the other hand, the assessment of the capacity of CTLA-4-targeting ligands to induce an “activating” CTLA-4 oligomer allows one to identify those immunomodulatory biologicals with inverse agonist properties, thus throwing a note of caution in their further therapeutic development and, possibly, avoiding the devastating effects recently seen with the administration of soluble CD28 superagonistic Abs.

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Disclosures
The authors have no financial conflict of interest.

References


