

A Major Costimulatory Molecule on Antigen-presenting Cells, CTLA4 Ligand A, Is Distinct from B7

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Summary

CTLA4 ligands are important costimulatory molecules because soluble CTLA4Ig blocks the induction of T cell responses and induces T cell tolerance. As CTLA4 immunoglobulin (CTLA4Ig) binds B7 when the latter is expressed on fibroblasts, it was widely assumed that CTLA4Ig blocks T cell costimulation by blocking the function of B7. Here we show that the major costimulatory ligand bound by CTLA4Ig (which we term CTLA4 ligand A) on antigen-presenting cells are not encoded by the B7 gene. CTLA4 ligand A also differs from B7 in cellular distribution and in the respective levels of expression. Both B7 and CTLA4 ligand A are critically involved in T cell costimulation.

Costimulatory molecules are required for induction of T cell clonal expansion and for prevention of T cell unresponsiveness. Numerous experiments have demonstrated that the costimulatory molecules that bind to CTLA4 play a major role in T cell costimulation. Thus, the chimeric molecule CTLA4Ig, which is composed of the extracellular domain of CTLA4 and the Fc fragment of Ig (1), inhibits T cell responses to anti-CD3 (2), allogeneic MHC (2, 3), and soluble proteins (2). In vivo, CTLA4Ig has been shown to inhibit T cell-dependent Ab responses (4) and to prolong survival of allogeneic and xenogeneic grafts (5, 6). More interestingly, CTLA4Ig induces specific T cell hyporesponsiveness to allogeneic antigen if T cells are stimulated with the allogeneic antigen in the presence of CTLA4Ig (7). In addition, CTLA4Ig induces tolerance for xenogeneic pancreatic grafts (5). Thus the ligand(s) for CTLA4Ig is critical both for induction of the immune response and for prevention of inactivation of T cells.

CTLA4 can bind to B7/BB1 (hereafter called B7) if the latter is expressed on fibroblasts (1). Because of this, it is widely assumed that the molecule that CTLA4Ig blocks on APC is B7 (1–8). However, the ligand CTLA4 on APC has not been well characterized. As multiple ligands have been shown to bind to one cell surface molecule (9, 10), the costimulatory molecule that binds to CTLA4 may not necessarily be B7. Here we report that the major ligand on APC for CTLA4 (hereafter called CTLA4 ligand A) is distinct from B7. Both B7 and the CTLA4 ligand A are expressed on APC and are critical for costimulation for CD4 T cell proliferation.

Materials and Methods

Production of Anti-B7 mAbs. Antimurine B7 mAbs were generated by fusion of murine myeloma Ag8.653 with spleen cells from hamsters that were immunized with Chinese hamster ovary (CHO)-mB7 three times at 2-wk intervals. The hybridoma supernatants were screened for their ability to bind CHO-mB7 but not CHO cells by flow cytometry.

Proliferation of Purified T Cells. CD4 T cells were purified from nonadherent spleen cells from naive CBA/CAJ mice by two rounds of treatment of complement and a cocktail of Abs, including anti-Thy1.2 mAb HO13.4.9 (11), anti-CD8 mAb M31 (12), anti-Mac-1 mAb M1/70.15.11.5H (13), and anti-heat stable antigen (HSA) mAb J11d (14). The CD4 T cells were stimulated with anti-CD3 mAb 2C11.145 (15) supernatants (1:40) and one of the three types of accessory cells: COS cells transfected with FcR2 (16) and B7, and T-depleted spleen cells, all pretreated with mitomycin C, as described (17). After 42 h of culture, the cells were pulsed with 1 μ Ci/well of [³H]TdR for an additional 6 h. The data shown were means of duplicates, with variations <15%.

Analysis of B7 mRNA by PCR. mRNA were prepared from 5×10^6 of either M12.4.1, CH27, or total spleen cells by the fast-track poly A RNA preparation kit (Clontech, Palo Alto, CA). First strand cDNA were prepared using 50 ng of hexamer random primers, 1 μ g mRNA, 200 U Moloney MuLV reverse transcriptase (GIBCO BRL, GA: MD). Similar results were obtained when poly dT or B7 reverse primer were used to prepare the first strand cDNA. The B7 or glyceraldehyde-3-phosphate dehydrogenase DNA were amplified from the cDNA by PCR reaction as has been described. The primers used were: B7 forward primer GAAGCTATGGCT-TGCAATTGTCAG, B7 reverse primer AGAAGAATAAAGGA-AGACGGTCT, GAPDH forward primer ATGGTGAAGGTCGGT-

GTGAACGGATTGGC, and GAPDH reverse primer CATCGA-AGGTGGAAGAGTGGGAGTTGCTGT.

Blocking of CTLA4Ig Binding by Anti-B7 mAb 3A12. CHO-mB7, LPS-activated B cells and spleen adherent cells prepared as described were incubated with a given concentration of anti-B7 mAb 3A12 (this study) for 30 min at 4°C. The cells were then incubated with CTLA4Ig (2 µg/ml) for 30 min. The binding of CTLA4Ig was detected by FITC-labeled goat anti-hamster IgG (Tago, Inc., Burlingame, CA) diluted in 1 mg/ml of normal hamster Ig. The data shown, percent maximal binding, was the percentage of CTLA4Ig binding in the absence of blocking Ab, as calculated by the mean fluorescence channel numbers. Goat anti-human IgG diluted with 1 mg/ml of hamster Ig did not bind 3A12 significantly, and this binding has been subtracted.

Results and Discussion

To address whether B7 is the major ligand for CTLA4 on APC, we have generated three antimurine B7 mAbs, 1D5, 3A12, and 7A5. All three mAbs bind B7 and block its costimulatory activity (Fig. 1). We have compared the binding of CTLA4 to normal spleen cells and LPS-activated B cells with that of anti-B7 mAbs. CTLA4Ig gives low but detectable binding to normal spleen cells, whereas three anti-B7 mAbs fail to bind spleen cells (Fig. 2a). For activated B cells, CTLA4Ig gives much better binding than the anti-B7 mAbs. As measured by the peak fluorescence channel numbers, a saturating amount of CTLA4Ig binds LPS-activated B cells about 20 times as well as anti-B7 mAb (Fig. 2b), although

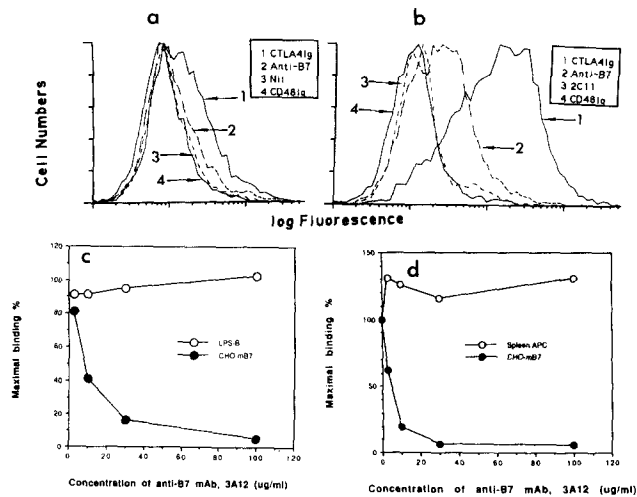


Figure 2. Comparative study of B7 and CTLA4 ligand by flow cytometry. (a) Binding of CTLA4Ig and anti-B7 mAbs to CBA/CaJ spleen cells. The cells were incubated with either CTLA4Ig (0.5 µg/ml, 500,000 cells/well in 50 µl), CD48Ig, or a cocktail of three anti-B7 mAbs (3A12, 1D5, and 7A5). The binding of CTLA4Ig was detected with FITC-labeled goat anti-human IgG (Accurate Chemical and Scientific Corp., Westbury, NY), whereas the binding of anti-B7 mAbs was detected with goat anti-hamster Ig (Caltag Laboratories, South San Francisco, CA). (b) Binding of anti-B7 mAb 3A12 (10 µg/ml) and CTLA4Ig (2 µg/ml) to LPS-activated B cells. Similar results were obtained with mAb 1D5. (c) Blocking of CTLA4Ig binding to CHO-mB7 and LPS-activated B cells by 3A12. (d) Blocking of the binding of CTLA4Ig to low density, spleen-adherent cells. This population contains 35% B220⁺ cells, 36% Mac-1⁺ cells, 32% CD11c⁺ cells, and 7% Thy1⁺ cells.

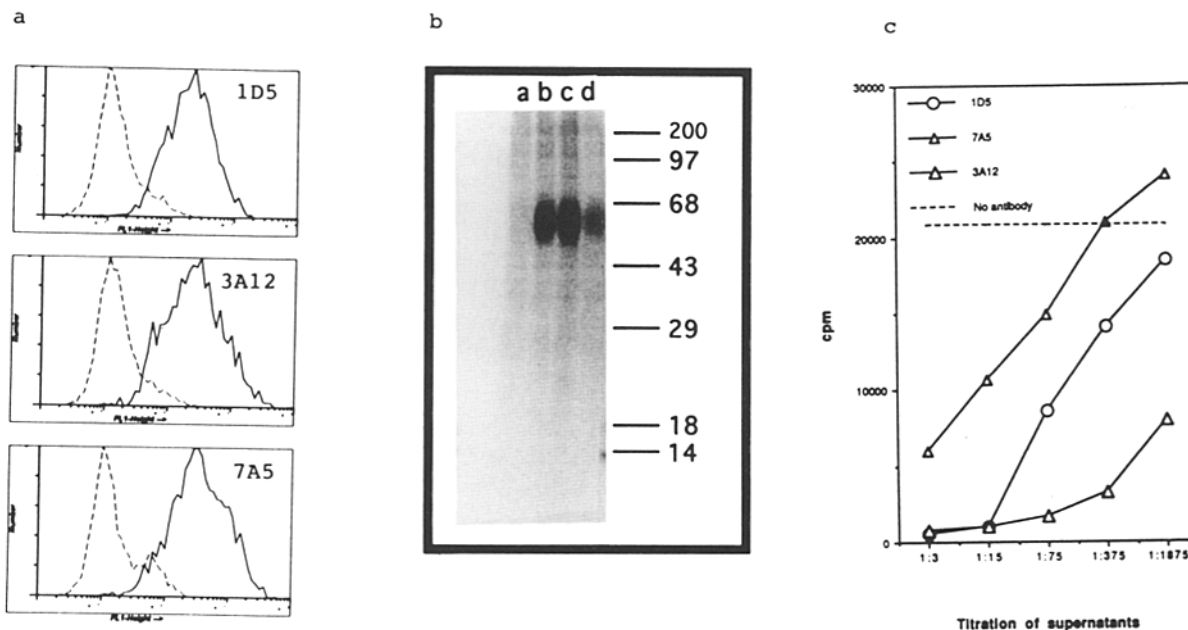


Figure 1. Characterization of hamster antimurine B7 mAbs. (a) mAbs 1D5, 3A12, and 7A5 bind to murine B7-transfected (CHO-mB7) but not untransfected CHO cells, as analyzed by flow cytometry. (b) Immunoprecipitation analysis of ¹²⁵I-labeled CHO-mB7 cell lysates with antimurine B7 mAbs. (lane a) Hamster mAb 21F10; (lane b) 1D5; (lane c) 3A12; and (lane d) 7A5. 7A5 was used as hybridoma supernatant, whereas other Ig were used at 3 µg/sample. (c) Antimurine B7 mAbs block the costimulatory activity of murine B7-transfected COS cells. COS cells (10,000/well) transfected with murine B7, FcR2, and B7 + FcR2 are used as accessory cells for CBA/CaJ CD4 T cells (100,000) stimulated with 1:40 dilution of anti-CD3 mAb 2C11.145 supernatant.

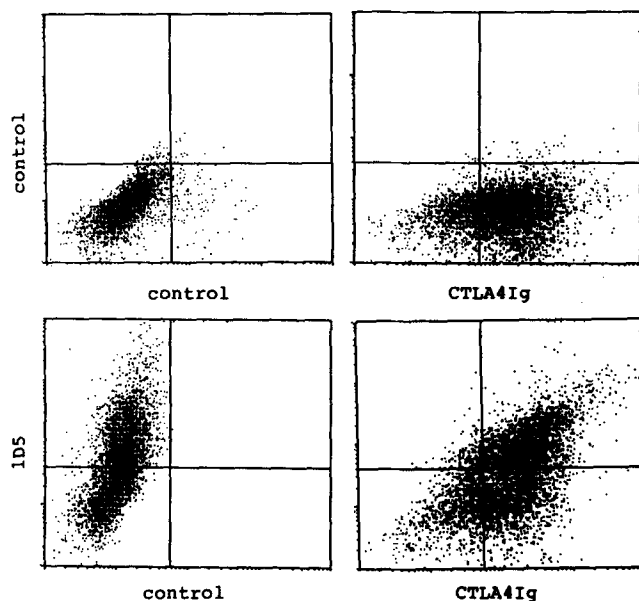


Figure 3. Two-color flow cytometry analysis of the distribution of B7 and CTLA4Ig ligand on LPS-activated B cells. LPS-activated B cells were dually stained with biotinylated anti-B7 mAb 1D5 and unlabeled CTLA4Ig. The binding of anti-B7 mAb was detected by PE-labeled avidin, and that of CTLA4Ig detected by FITC-labeled goat anti-human IgG. Similar results were obtained with mAb 3A12.

both reagents used at saturating concentrations bind CHO cells transfected with B7 comparably (data not shown). Anti-B7 mAb 3A12 blocks CTLA4 binding to CHO-mB7, but not to LPS-activated B cells (Fig. 2 *c*), or to low-density spleen adherent cells (Fig. 2 *d*). This differential blocking of CTLA4Ig binding by anti-B7 mAb on different cell types indicates that the majority of the CTLA4 ligand on APC is distinct from B7 expressed on CHO cells. Furthermore, anti-B7 mAbs bind only a fraction of LPS-activated B cells that bind CTLA4Ig (Fig. 3). Thus, among activated B cells, some cells only express a non-B7 CTLA4Ig ligand, which we call CTLA4 ligand A.

Cell surface iodination labels CTLA4 ligand on CHO-mB7 but not on LPS-activated B cells as assayed by immunopre-

cipitation (data not shown). As iodination requires tyrosine or histidine in the extracellular domain, this result raises the possibility that the amino acid composition of CTLA4Ig ligand on LPS-activated B cells may be different from B7. In other words, CTLA4 ligand A may be encoded by a non-B7 gene. Our analysis of B7 mRNA and cell surface expression of B7 and CTLA4 ligands on a panel of B lymphoma cell lines demonstrates that this is the case. The results of two representative cell lines are shown in Fig. 4. CH27 binds both anti-B7 and CTLA4Ig. M12.4.1, on the other hand, binds CTLA4Ig but not anti-B7 (Fig. 4 *a*). This lack of binding to anti-B7 mAbs was due to a lack of the expression of B7 molecules, as no mRNA for B7 could be detected from M12.4.1 by PCR (Fig. 4 *b*) using three different methods to prepare the first-strand cDNA. However, B7 mRNA was easily detected from CH27 and spleen cells. Careful titration over 1,000-fold range of RNA concentration gives the same results (data not shown). Thus CTLA4Ig binds to a cell line that does not express B7 gene. These results clearly demonstrate that CTLA4 can bind to a ligand other than B7. These results also rule out the possibility that the distinction of B7 and CTLA4 ligand A revealed by binding studies was due to posttranslational modification of B7.

To test the function of CTLA4 ligand A, we compared the costimulatory activity of M12.4.1, which expresses CTLA4 ligand A but not B7, with CH27 which expresses both B7 and CTLA4 ligand A. The accessory cells were fixed before addition to T cells in order to prevent induction of B7 or CTLA4 ligand A on the accessory cells by T cells (18–20). As shown in Fig. 5 *a*, both M12.4.1 and CH27 have costimulatory activity for CD4 T cells. The costimulatory activity of M12.4.1 was blocked completely by CTLA4Ig but not by anti-B7 mAb 3A23 (Fig. 5 *b*). These results demonstrate that CTLA4 ligand A is critical for costimulatory activity and that CTLA4 ligand A can costimulate T cell proliferation in the absence of B7. To estimate the contribution of B7 and CTLA4 ligand to the costimulatory activity of spleen cells, we compared anti-B7 mAb 3A12 with CTLA4Ig in inhibiting CD4 T cell proliferation. 3A12 is comparable with CTLA4 Ig in inhibiting the costimulatory activity of B7 expressed on COS cells (Fig. 5 *c*). However, when spleen APC are used,

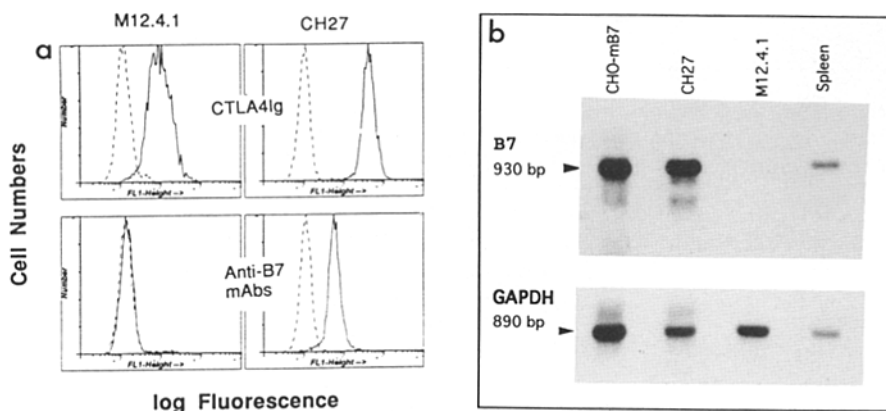


Figure 4. M12.4.1 cells express no B7 mRNA but express CTLA4 ligand A. (a) M12.4.1 or CH27 were incubated with either CTLA4Ig (top) or anti-B7 mAb cocktails (bottom) and their binding detected by FITC-labeled goat anti-human IgG or goat anti-hamster IgG. (Dashed lines) FACS[®] profiles of B cells incubated with no primary Ab; (solid lines) those incubated with specific mAbs or chimeric molecules. (b) PCR analysis of the expression of B7 genes in spleen cells, CH27 and M12.4.1. The GAPDH primers were used as internal controls. PCR products were detected by Southern blot using ³²P-labeled full-length B7 cDNA, or ³²P-labeled GAPDH PCR products.

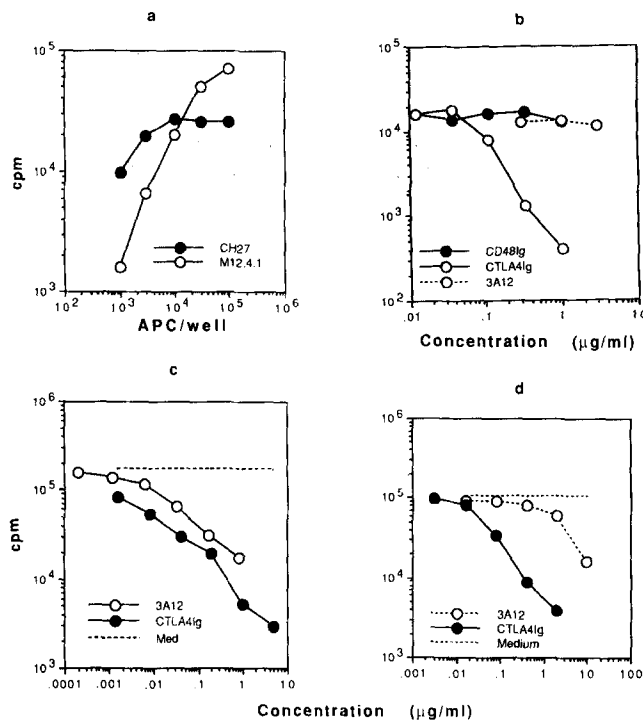


Figure 5. Contribution of CTLA4 ligand A to costimulation for CD4 T cell proliferation. (a) Comparison of the costimulatory activity of M12.4.1 and CH27. Varying numbers of fixed CH27 or M12.4.1 cells were incubated with 5×10^4 /well of CD4 T cells and soluble anti-CD3 mAb 2C11.145. (b) Inhibition of the costimulatory activity of M12.4.1 by CTLA4Ig. 5×10^4 /well of CD4 T cells were stimulated with soluble anti-CD3 mAb in the presence of 10^4 /well of M12 cells as accessory cells. Varying concentrations of CTLA4Ig, anti-B7 mAb 3A12, and CD48Ig were added at the beginning of the culture. (a and b) Proliferative response of CD4 T cells were determined by incorporation of [³H]TdR into T cells at 72 h of culture. (c and d) Inhibition of CD4 T cell proliferation with anti-B7 mAb 3A12 and CTLA4Ig. 10^4 /well of COS cells transfected with B7 and FcRII (c) or 10^5 /well of T-depleted spleen cells (d) were used as accessory cells. In both c and d, the accessory cells were treated with mitomycin C (100 μg/ml, for 1 h at 37°C) before addition to the culture and the proliferative responses were determined at 48 h of culture.

CTLA4Ig, which binds both B7 and CTLA4 ligand A, is about 50–100-fold more efficient in blocking T cell costimulation (Fig. 5 d).

These data, taken together, revealed that CTLA4 ligand A is the major ligand on APC and is expressed independently

of the B7 gene. Both CTLA4 ligand A and B7 are critically involved in costimulating T cell proliferation. Our results reconcile the widely experienced differences in activity between anti-B7 mAbs and CTLA4Ig (19–22) without invoking a higher affinity of CTLA4Ig (1).

Our conclusion has several important implications for understanding the molecular basis of T cell costimulation. First, much of the function of B7 as deduced from blocking studies by CTLA4Ig (1–8) is likely contributed by both B7 and CTLA4 ligand A. The function of B7 as the major costimulator molecule needs to be reevaluated. Second, although both B7 and CTLA4Ig ligand are involved in T cell costimulation, it is not clear at this point whether they serve different functions in T cell costimulation. Whereas B7 binds both CTLA4 and CD28, it is not known whether CTLA4 ligand A binds CD28, although circumstantial evidence suggests that CD28 may also bind to CTLA4 ligand A. Thus, anti-B7 mAbs only partially block the binding of lymphocytes to CHO cells transfected with CD28 (23). However, the same Abs can completely inhibit the binding of B7 to CD28 (8). Furthermore, M12 which express CTLA4 ligand A but not B7, also enhances the IL-2 production by CD4 T cells (data not shown), suggesting that CTLA4 ligand A may have a function similar to that documented for B7 (8). Freshly isolated spleen cells do not express any detectable amount of B7, although a low level CTLA4 ligand A is detected. The expression of CTLA4 ligand A on unstimulated APC means that CTLA4 ligand A may play a critical role in the initiation of T cell responses, such as preventing clonal anergy of T cells. B7 may play a more critical role in amplification of T cell responses. Finally, when professional APC are used, multiple costimulatory molecules, such as B7 (1–8), CTLA4 ligand A (this study), and HSA (2, 17) are needed for T cell responses, because Abs or soluble receptors to any of these molecules have been shown to inhibit T cell proliferation. On the other hand, cells that express only one of these molecules have been shown to costimulate T cell proliferation. This discrepancy can be explained by the amount of the costimulators expressed, the structure of the costimulator, and the kinetics of expression of the costimulatory molecules in different cell types. The existence of multiple costimulators on APC offers flexibility in regulating costimulatory activity of APC.

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