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Immunology

Evidence for an additional ligand, distinct from B7, for the CTLA-4 receptor

ZIBA RAZI-WOLF*, FRANCES GALVIN*, GARY GRAY†, AND HANS REISER*‡

*Division of Lymphocyte Biology, Dana-Farber Cancer Institute, Boston, MA 02115; and †Repligen Corporation, Cambridge, MA 02139

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ABSTRACT **Activation of T lymphocytes requires the recognition of peptide–major histocompatibility complex complexes and costimulatory signals provided by antigen-presenting cells (APCs). The best-characterized costimulatory molecule to date is the B7 antigen, a member of the immunoglobulin family that binds two receptors, CD28 and CTLA-4, expressed on the T-cell surface. Using the anti-mouse B7 (mB7) monoclonal antibody (mAb) 16-10A1, which we recently developed, we found that mB7 is indeed an important costimulatory ligand for the antigen-specific activation of murine T cells by B lymphocytes. Three lines of evidence suggest, however, the existence of at least one additional ligand for the CTLA-4 receptor. First, a soluble fusion protein of human CTLA-4 and the IgG1 Fc region, termed CTLA4Ig, blocks better than the anti-mB7 mAb the allogeneic stimulation of T cells by unfractionated splenic APCs. Second, saturating amounts of anti-mB7 mAb do not significantly block binding of fluorescein isothiocyanate-conjugated CTLA4Ig to activated splenic APCs. Furthermore, CTLA4Ig but not the anti-mB7 mAb reacts with the M12 and M12.C3 cell lines. The identification of an additional ligand for CTLA-4 may have applications to the treatment of autoimmune disease and transplant-associated disorders.**

Activation of CD4+ T cells occurs upon recognition, by the T-cell receptor (TCR), of peptide–major histocompatibility complex complexes (MHC), which are expressed on the surface of an antigen-presenting cell (APC). TCR triggering, however, is not sufficient to impart complete T-cell activation. The induction of lymphokine secretion and cell proliferation requires a second, costimulatory signal, which is also provided by an APC (1, 2).

The CD28 molecule is the best-characterized costimulatory receptor. Binding of monoclonal antibodies (mAbs) to CD28 (3), a 44-kDa homodimeric T-cell glycoprotein that is a receptor for B7 (4), can augment the proliferation or lymphokine secretion of T cells stimulated with anti-CD3 mAb or with antigen (5–8) and overcome T-cell anergy in T-cell clones (8). CTLA-4 is an additional TCR with affinity for B7, but the function of CTLA-4 in T-cell activation remains to be determined (9–12).

We and others have shown that B7 (13–20) can provide a costimulatory signal for CD4+ T cells (20–25), but it is currently unknown whether there exist additional ligands, besides B7, with affinity for the CD28 and CTLA-4 receptors. In the present report, we have addressed this issue in the murine system, using the anti-mouse (mB7) mAb 16-10A1 (18) and CTLA4Ig, a soluble fusion protein between human CTLA-4 and an Igγ constant region, which inhibits T-cell activation *in vitro* (10, 26) and *in vivo* (27–29). This reagent binds both human B7 and mB7 and has the potential to detect additional ligands besides B7 with affinity for the CD28 and CTLA-4 receptors. In the present report, we provide evidence that there exists indeed at least one additional ligand, distinct from B7, with affinity for the CTLA-4 receptor.

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MATERIALS AND METHODS

Antigens and Fusion Proteins. Rabbit anti-mouse immunoglobulin (RAMG) was obtained from rabbits immunized with normal mouse immunoglobulin and was purified on normal mouse immunoglobulin-conjugated Sepharose columns. $F(ab')_2$ fragments of RAMG were prepared by pepsin digestion using a commercially available kit (Pierce). The fusion protein CTLA4Ig, composed of the extracellular domains of human CTLA-4 and the hinge–CH2–CH3 domains derived from a human IgG1 gene, was derived by polymerase chain reaction using a strategy similar to that previously reported (10). The genetic fusion was expressed in CHO cells, and the fusion protein was purified from culture supernatants.

Purification of Murine Spleen Cells. Splenic T cells were purified by nylon wool enrichment (30). CD4+ T cells were isolated as described (31, 32). T-cell-depleted spleen cells were purified as described (18). Small resting B cells were purified from the spleens of resting BALB/c mice as described by Krieger *et al.* (33). Where indicated, APCs were stimulated for 3 days in culture medium (24) supplemented with lipopolysaccharide (LPS) (10 μg/ml) (Difco) and dextran sulfate (20 μg/ml) (Pharmacia).

Cell Cultures. Microcultures were set up in duplicate as described (18). The precise culture constituents and incubation periods are described in the respective experimental protocols. All cultures were pulsed with 1 μCi of [^{3}H]thymidine per well (1 Ci = 37 GBq) for the last 6–8 hr of the incubation period to assay for T-cell proliferation. To assay for lymphokine secretion, 100 μl of culture supernatant was harvested 36 hr after initiation of the culture. Interleukin 2 (IL-2) content was assayed on the HT-2 indicator cell line as described (24). IL-4 content was determined with a commercially available ELISA kit (Endogen, Boston, MA).

RESULTS

Role of mB7 in B-Cell-Dependent T-Cell Activation. Experiments with purified B lymphocytes revealed that mB7 was important for the antigen-specific and MHC-restricted activation of T cells by B cells. We examined initially whether the anti-mB7 mAb 16-10A1 (18) and CTLA4Ig were able to inhibit alloreactive T-lymphocyte responses initiated by LPS/dextran sulfate-stimulated purified B lymphocytes. Purified C57BL/6 CD4+ T cells and BALB/c B-cell blasts were incubated in the presence of medium, anti-mB7 mAb,

Abbreviations: APC, antigen-presenting cell; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; mB7, murine B7; LPS, lipopolysaccharide; MHC, major histocompatibility complex; TCR, T-cell receptor; RAMG, rabbit anti-mouse immunoglobulin; IL, interleukin.

‡To whom correspondence should be addressed at: Division of Lymphocyte Biology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

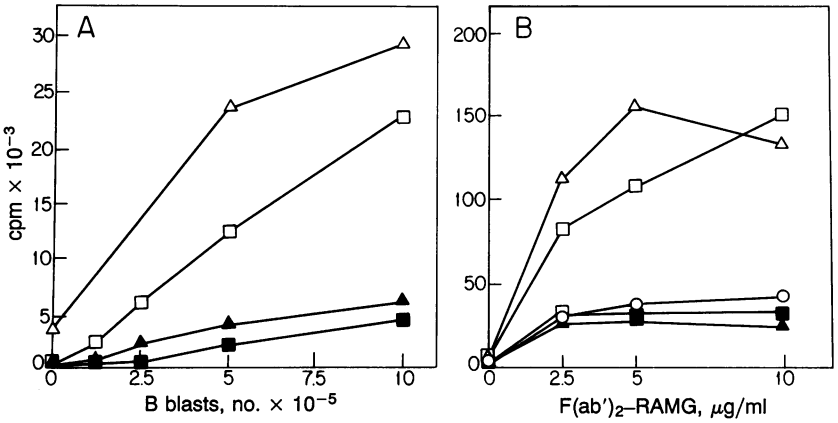

FIG. 1. mB7 is an important costimulatory molecule in B-cell-dependent T-cell activation. (*A*) Anti-mB7 mAb and CTLA4Ig block activation of T cells induced by allogeneic B cells. Microcultures were prepared with 2 × 10^5 C57BL/6 CD4$^+$ T lymphocytes. Indicated on the *x* axis is the number of irradiated (1500 rad; 1 rad = 0.01 Gy) B-cell blasts added to the culture wells. Medium (□), anti-mB7 mAb 16-10A1 (2 μg/ml) (■), CTLA4Ig (2 μg/ml) (▲), or HP25 mAb (2 μg/ml) (△) was added as indicated. All B cells were prestimulated with LPS and dextran sulfate before irradiation and addition to the cultures. (*B*) Anti-mB7 mAb blocks activation of T cells by RAMG presented by syngeneic purified resting B cells. BALB/c mice were primed s.c. with 100 μg of RAMG in complete Freund's adjuvant. Two weeks later, regional lymph nodes were harvested. T cells were isolated by 2-fold treatment with anti-MHC class II mAb and C′. Microcultures were prepared with 2 × 10^5 T lymphocytes and either medium (○) or 2 × 10^5 mitomycin C-treated B cells (□, ■, ▲, △) that had been purified as described. Where indicated, anti-mB7 mAb 16-10A1 (2 μg/ml) (■), CTLA4Ig (4 μg/ml) (▲), or HP25 mAb (2 μg/ml) (△) was added to the cultures. Indicated on the *x* axis are concentrations of F(ab′)$_2$–RAMG added to the culture wells. Cultures were pulsed after 5 days (*A*) or 4 days (*B*) with 1 μCi of [^{3}H]thymidine per well for the last 8 hr of the incubation period to assay for T-cell proliferation.

CTLA4Ig, or control mAb HP25 (18). After 5 days, cultures were pulsed with [^{3}H]thymidine to assay for T-cell proliferation. A representative experiment is shown in Fig. 1*A*. As expected, activated BALB/c B cells stimulated the proliferation of purified C57BL/6 CD4$^+$ T cells. This stimulatory effect is clearly mB7 dependent, as it is inhibited by the addition of either anti-mB7 mAb or CTLA4Ig. The HP25 control mAb did not display an inhibitory effect.

Next we analyzed whether mB7 played a role in the antigen-specific activation of murine T cells by B cells normally initiated by B lymphocytes after uptake of antigens through their cell surface antigen receptors. For this purpose, BALB/c mice were primed *in vivo* with RAMG as a source of antigen-specific T cells. Two weeks later, the animals were sacrificed and their regional lymph nodes were harvested. Cultures were constructed with purified CD4$^+$ T cells as detailed in the legend to Fig. 1*B*. Restimulation of T cells with an F(ab′)$_2$ preparation of RAMG as source of antigen did not induce a T-cell response in the absence of B cells, whereas the T cells proliferated when incubated with F(ab′)$_2$–RAMG and mitomycin C-treated B cells (Fig. 1*B*). Most importantly, addition of anti-mB7 mAb or CTLA4Ig significantly inhibited T-cell proliferation induced by F(ab′)$_2$–RAMG. The control mAb HP25 did not display significant functional effects (Fig. 1*B*).

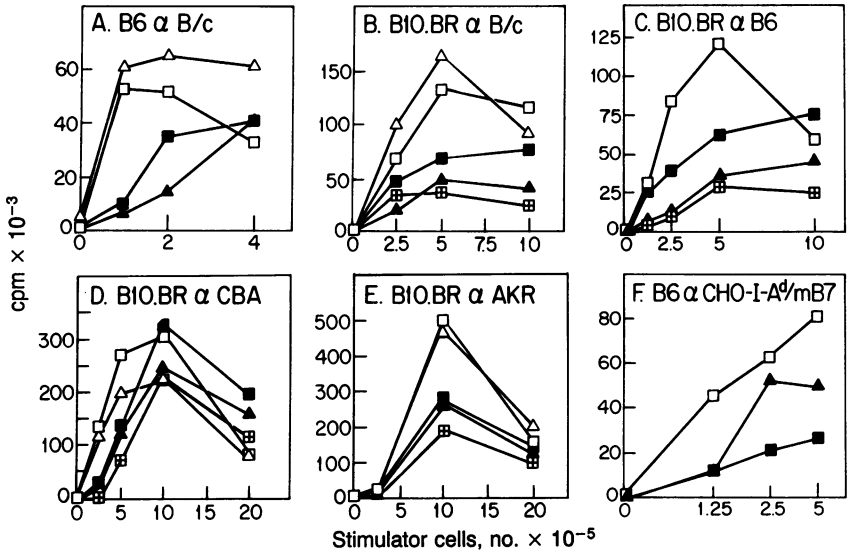

FIG. 2. Functional effects of anti-mB7 mAb and CTLA4Ig on the activation of T cells induced by unfractionated, mitomycin C-treated spleen cells. Microcultures were prepared with 2 × 10^5 T lymphocytes and unfractionated mitomycin C-treated spleen cells. The following strain combinations were analyzed (T-cell anti-APCs): C57BL/6 anti-BALB/c (*A*), B10.BR anti-BALB/c (*B*), B10.BR anti-C57BL/6 (*C*), B10.BR anti-CBA/J (*D*), B10.BR anti-AKR/J (*E*). (*F*) Allospecific response of C57BL/6 T cells, induced by mitomycin C-treated CHO-I-A^d/mB7 cells, was analyzed. Medium (□), anti-mB7 mAb 16-10A1 (2 μg/ml) (■), CTLA4Ig (4 μg/ml) (▲), HP25 mAb (2 μg/ml) (△), or a combination of anti-mB7 mAb and CTLA4Ig (⊞) was added as indicated. Indicated on the *x* axis is the number of mitomycin C-treated stimulator cells added to the culture wells. Cultures were pulsed after 5 days with 1 μCi of [^{3}H]thymidine per well for the last 6 hr of the incubation period to assay T-cell proliferation.

Effects of Anti-mB7 mAb and CTLA4Ig on Alloresponses Induced by Unfractionated Spleen Cells. Our studies on the existence of additional ligands for the CD28 and CTLA-4 receptors were prompted by additional blocking studies using both the anti-mB7 mAb and the CTLA4Ig reagent. Briefly, these reagents were assayed for their ability to inhibit allomixed lymphocyte reactions induced by unfractionated spleen cells. For this purpose, nylon wool-purified T cells from either the B6 or the B10.BR mouse strains were incubated with mitomycin C-treated spleen cells from various mouse strains. Representative experiments are shown in Fig. 2 *A–E*. Our results can be summarized as follows: (*i*) the blocking by anti-mB7 mAb and CTLA4Ig was quantitatively variable; nevertheless, some degree of blocking was observed with either reagent in most strain combinations studied. (*ii*) Interestingly, relatively poor blocking was consistently observed in the B10.BR anti-CBA combination, although some blocking was seen at lower numbers of CBA stimulator cells. This raises the possibility that another costimulatory ligand is involved in the induction of this response. (*iii*) CTLA4Ig was either a better blocker or equally efficient when compared to anti-mB7 mAb (Fig. 2 *A–E*). In contrast, the anti-mB7 mAb was more efficient at inhibiting alloresponses induced by transfected CHO-I-A^d/mB7 cells (Fig. 2*F*; data not shown).

Similar results were obtained when IL-2 production was analyzed. For this purpose, we examined the allo-specific response of B10.BR T lymphocytes to BALB/c spleen cells (Fig. 3). Anti-mB7 mAb, CTLA4Ig, or a control mAb was added to the cultures. Thirty-six hours after initiation of the culture, supernatants were harvested and analyzed for IL-2 secretion in the HT-2 assay. The production of IL-4 was analyzed in an ELISA. As shown in Fig. 3*A*, both anti-mB7 mAb and CTLA4Ig blocked the secretion of IL-2. As observed above in the case of T-cell proliferation, CTLA4Ig was a better blocker when compared to anti-mB7 mAb. Interestingly, in contrast to their effect on the production of IL-2, neither of these reagents inhibited the secretion of IL-4. This finding raises the possibility that mB7 costimulation is not necessary for the activation of IL-4-secreting T cells, even though it can be sufficient for the activation of these cells (24, 34).

Reactivity of CTLA4Ig with LPS/Dextran Sulfate-Stimulated, T-Cell-Depleted Spleen Cells After Preincubation with Anti-mB7 mAb. To determine whether CTLA-4 recognized an antigen distinct from mB7, we asked if preincubation of activated APCs with anti-mB7 mAb could inhibit binding of CTLA4Ig to the surface of activated splenic APCs.

The feasibility of this approach is demonstrated in Fig. 4*A*. Both the anti-mB7 mAb and CTLA4Ig recognize mB7 on the surface of CHO cells transfected with mB7 (Fig. 4 *A1* and *A2*). Preincubation of CHO-mB7 cells with anti-mB7 mAb markedly inhibits binding of fluorescein isothiocyanate (FITC)-conjugated anti-mB7 mAb to the cell surface (Fig. 4 *A3* and *A5*) as determined by flow fluorocytometry. Most importantly, binding of FITC-labeled CTLA4Ig is also significantly blocked (Fig. 4 *A4* and *A6*). In parallel, we analyzed T-cell-depleted spleen cells stimulated with LPS and dextran sulfate (Fig. 4*B*). Both FITC-conjugated anti-mB7 mAb and FITC-conjugated CTLA4Ig recognize determinant(s) on these cells (Fig. 4 *B1* and *B2*). As observed with CHO-mB7 cells, preincubation of LPS/dextran sulfate blasts with anti-mB7 mAb markedly reduces binding of FITC-labeled anti-mB7 mAb to the cell surface (Fig. 4 *B3* and *B5*). In contrast, binding of FITC-conjugated CTLA4Ig was not significantly affected by preincubation of splenic APCs with anti-mB7 mAb at two different concentrations (Fig. 4 *B4* and *B6*). This result suggests the existence of antigen(s), distinct from mB7, recognized by CTLA4Ig.

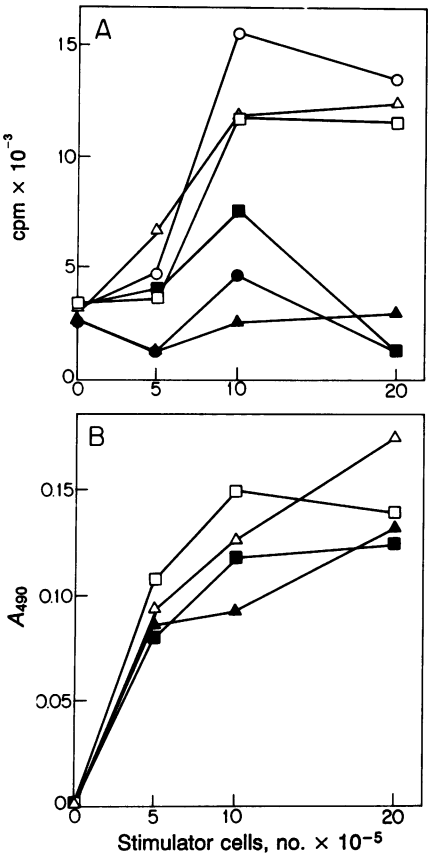

FIG. 3. Effect of anti-mB7 mAb and CTLA4Ig on production of IL-2 and IL-4. Microcultures were set up with 2×10^5 B10.BR $CD4^+$ T lymphocytes and unfractionated mitomycin C-treated BALB/c spleen cells (number of stimulator cells indicated on the *x* axis). Primary cultures contained either medium (□, ●, ○), anti-mB7 mAb 16-10A1 (2 μg/ml) (■), CTLA4Ig (4 μg/ml) (▲), or HP25 mAb (2 μg/ml) (△). Culture supernatants were harvested 36 hr after initiation of the culture and assayed in parallel for IL-2 and IL-4 content. (*A*) Second incubation: IL-2 assay. IL-2 content was assayed with the IL-2-sensitive HT-2 indicator cell line, generously provided by Abul Abbas (Department of Pathology, Brigham and Women's Hospital and Harvard Medical School). Under the experimental conditions chosen, this cell line detects only IL-2 and no IL-4 as demonstrated through the respective blocking anti-IL-2 (●) and anti-IL-4 (○) mAbs. □, ■, △, and ▲, medium in the second incubation. HT-2 cells were incubated overnight and pulsed with 1 μCi of [^{3}H]thymidine per well for the last 6 hr of the incubation period to assay for HT-2 T-cell proliferation. (*B*) IL-4 assay. IL-4 content was determined with a commercially available ELISA kit (Endogen, Boston, MA). IL-4 determinations were carried out according to the manufacturer's conditions.

Reactivity of CTLA4Ig but Not Anti-mB7 mAb with Determinant(s) Expressed on the Surface of the M12 and M12.C3 Cell Lines. We have demonstrated that the B-lymphoblastoid cell line M12 (35) and its variant M12.C3 (36) can provide a costimulatory signal for the polyclonal activation of T cells (31). We have subsequently shown that M12 does not express mB7 on its cell surface and that the anti-mB7 mAb 16-10A1 does not inhibit the costimulatory activity of this cell line (18).

It was of interest to determine whether M12 cells expressed a determinant recognized by CTLA4Ig. To investigate this point, we examined the expression of cell surface antigens by immunofluorescence and flow fluorocytometry. As shown in Fig. 5*A*, the M12 cell line does not express mB7 as expected. In contrast, CTLA4Ig detects antigen(s) on M12 cells. Similarly to M12, M12.C3 cells are mB7 negative (Fig. 5*C*) but express a determinant reactive with CTLA4Ig (Fig. 5*D*).

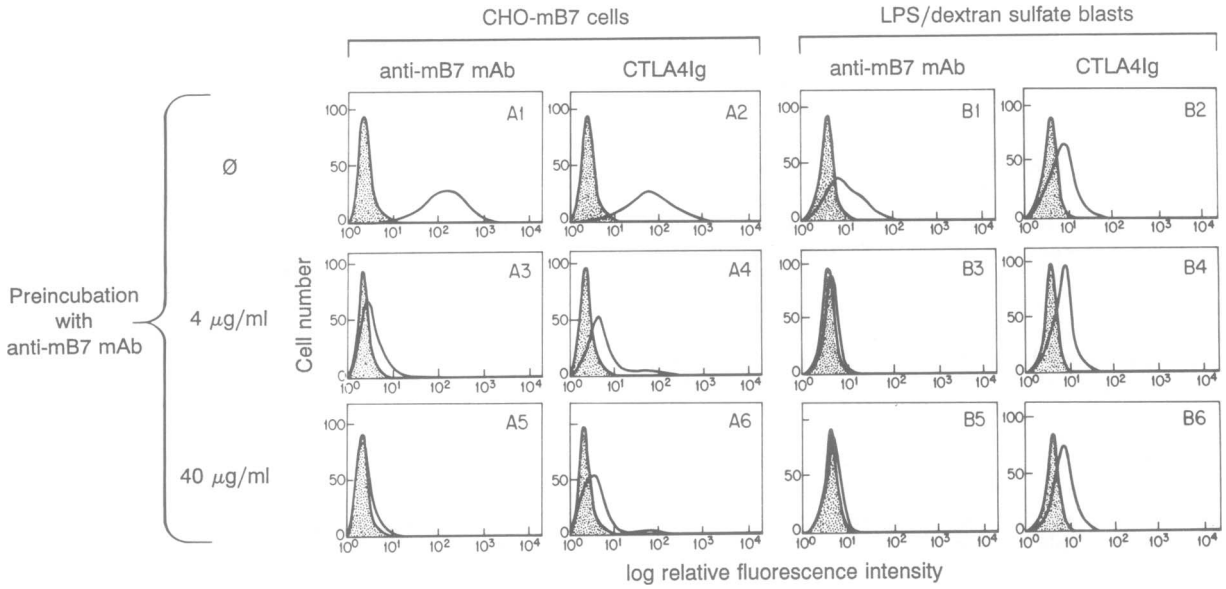

FIG. 4. Reactivity of CTLA4Ig with LPS/dextran sulfate-stimulated APCs after preincubation with anti-mB7 mAb. CHO-mB7 cells (*A*) or LPS-dextran sulfate-stimulated splenic APCs (*B*) were either mock incubated (*A1*, *A2*, *B1*, and *B2*) or incubated with anti-mB7 mAb 16-10A1 at 4 μg/ml (*A3*, *A4*, *B3*, and *B4*) or 40 μg/ml (*A5*, *A6*, *B5*, and *B6*), all for 30 min on ice. At the end of the incubation, cells were spun down, washed once in medium, and analyzed by immunofluorescence with FITC-labeled anti-mB7 mAb (*A1*, *A3*, *A5*, *B1*, *B3*, and *B5*) or FITC-labeled CTLA4Ig (*A2*, *A4*, *A6*, *B2*, *B4*, and *B6*). Control stainings were carried out with FITC-conjugated anti-I-$A^{k,s}$ mAb OX6 (GIBCO/BRL) (stippled area).

DISCUSSION

To determine whether mB7 was necessary for antigen-specific immune responses and whether there exist additional ligands for the CD28 and CTLA-4 receptors, we studied the effect of two different anti-mB7 reagents on murine T-cell responses to purified B cells or heterogeneous populations of splenic APCs. Our findings confirm and extend previous studies. We initially demonstrated that the mB7 molecule is an important costimulatory ligand in B-cell-dependent T-cell activation. Both the anti-mB7 mAb and CTLA4Ig efficiently blocked a primary mixed lymphocyte reaction induced by activated allogeneic B lymphocytes. These experiments extend previous studies in the human system by Koulova *et al.* (37) and Linsley *et al.* (10) with Epstein–Barr virus-transformed B-cell blasts. In addition, both anti-mB7 reagents inhibited the presentation of a model antigen, RAMG, which has been processed through the receptor-mediated pathway of B-cell antigen presentation to RAMG-specific T cells.

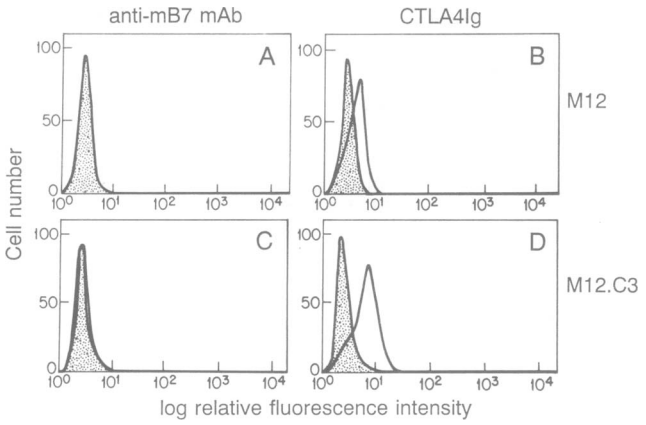

FIG. 5. Reactivity of CTLA4Ig but not anti-mB7 mAb with determinant(s) expressed on the surface of the M12 and M12.C3 cell lines. M12 (*A* and *B*) and M12.C3 (*C* and *D*) cells were incubated with FITC-labeled anti-mB7 mAb (*A* and *C*) or FITC-labeled CTLA4Ig (*B* and *D*). Control stainings to obtain background fluorescence values were performed with FITC-conjugated anti-I-$A^{k,s}$ mAb OX6 (stippled area).

In the present report, we provide three lines of evidence for an additional ligand, distinct from mB7 for the CTLA-4 receptor. (*i*) Although both anti-mB7 mAb and CTLA4Ig can inhibit allospecific T-cell responses induced by unfractionated splenic APCs, CTLA4Ig is a more efficient blocking reagent in most experiments. In contrast, anti-mB7 mAb is more efficient at blocking an allo-mixed lymphocyte reaction induced by MHC class II- and mB7-transfected CHO cells. Thus, CTLA4Ig does not simply have a higher affinity for the B7 molecule than the anti-mB7 mAb. (*ii*) Preincubation of activated APCs with saturating amounts of anti-mB7 mAb does not significantly inhibit binding of FITC-conjugated CTLA4Ig to the cell surface. (*iii*) We have identified two cell lines, M12 and M12.C3, that express a determinant(s) recognized by CTLA4Ig but do not express mB7 on their cell surface.

Two additional points should be noted in the context of these findings. (*i*) Our studies are based on the differential reactivity of anti-mB7 mAb and the CTLA4Ig fusion protein. Given that B7 binds to receptors on the T-cell surface, CD28 and CTLA-4, it seems likely that this costimulatory APC molecule is also capable of binding CD28. (*ii*) We have not yet been able to define the molecule recognized by CTLA4Ig by immunoprecipitation. Our evidence for the existence of this molecule is therefore only indirect at the moment.

Although our findings strongly suggest that the additional ligand(s) for CTLA-4 is functional, we have observed several situations that strongly suggest the existence of other costimulatory molecules that do not bind the CTLA-4 receptor. First, we and others have shown that mB7 can costimulate both IL-2- and IL-4-secreting T cells (24, 34). In the present report, we show that neither anti-mB7 mAb nor CTLA4Ig inhibits IL-4 production. Recently, Tan *et al.* (38) have reported similar findings in the human system using CTLA4Ig as a blocking reagent. This finding suggests that another costimulatory ligand is responsible for the activation of IL-4-secreting T cells. In the case of at least some IL-4-

secreting T-cell clones, this costimulatory molecule is presumably IL-1 (2). Second, CTLA4Ig only inefficiently blocked the allo-mixed lymphocyte reaction B10.BR anti-CBA. These two mouse strains differ at the *Mls* locus (39). This finding raises the possibility that T-cell activation induced by some viral superantigens is not dependent on mB7 or other ligands for CD28/CTLA-4. This finding may have implications for the treatment of transplant-associated immune responses. In the context of our findings it is also noteworthy that Damle *et al.* (40) have recently reported that proliferation of human T lymphocytes induced by bacterial superantigens cannot be blocked by CTLA4Ig. Finally, we have previously shown that the costimulatory activity of M12 cells is not inhibited by anti-mB7 mAb (18). Preliminary experiments indicate that CTLA4Ig also does not inhibit the costimulatory activity of M12 and M12.C3 cells for Con A-induced T-cell activation (data not shown). This raises the possibility that there may exist additional costimulatory molecules concerned with T-cell activation that do not trigger through the CD28 and CTLA-4 receptors. One such ligand may be the heat-stable antigen (41).

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