Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11054–11058, December 1993
Immunology

Expression and functional significance of an additional ligand for CTLA-4

DEBORAH J. LENSCHOW*, GLORIA HUEI-TING SU*, LINDA A. ZUCKERMAN*, NASRIN NABAVI†, CINDY L. JELLIS‡, GARY S. GRAY‡, JIM MILLER*§, AND JEFFREY A. BLUESTONE*¶

*Committee on Immunology, and §Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637; †Roche Research Center, Nutley, NJ 07110; and ‡Repligen Corporation, Cambridge, MA 02139

Communicated by Josef Fried, August 30, 1993

ABSTRACT **Effective T-cell activation requires antigen/major histocompatibility complex engagement by the T-cell receptor complex in concert with one or more costimulatory molecules. Recent studies have suggested that the B7 molecule, expressed on most antigen presenting cells, functions as a costimulatory molecule through its interaction with CD28 on T cells. Blocking the CD28/B7 interaction with CTLA4Ig inhibits T-cell activation *in vitro* and induces unresponsiveness. We demonstrate that another molecule(s), termed B7-2, is expressed constitutively on dendritic cells, is differentially regulated on B cells, and costimulates naive T cells responding to alloantigen. B7-2 is up-regulated by lipopolysaccharide in <6 hr and is maximally expressed on the majority of B cells by 24 hr. In contrast, B7 is detected only on a subset of activated B cells late (48 hr) after stimulation. In addition, Con A directly induces B7-2 but not B7 expression on B cells. Finally, although both anti-B7 monoclonal antibodies and CTLA4Ig blocked T-cell proliferation to antigen-expressing B7 transfectants, only CTLA4Ig had any significant inhibitory effect on T-cell proliferation to antigens expressed on natural antigen presenting cells, such as dendritic cells. Thus, B7 is not the only costimulatory molecule capable of initiating T-cell responses since a second ligand, B7-2, can provide a necessary second signal for T-cell activation.**

Antigen-specific T-cell activation depends on T-cell receptor (TCR) interaction with peptide/major histocompatibility complex (MHC) in conjunction with costimulatory signals mediated by accessory molecules. In the absence of appropriate costimulation, T-cell clones are functionally inactivated or anergized, as defined by the failure to proliferate and produce lymphokines such as interleukin 2 (1, 2). Blocking one ligand pair, CD28 (expressed on the T cell) (3, 4) and B7 (5, 6) [expressed on dendritic cells (7), macrophages (8), and activated B cells (9, 10)], results in T-cell anergy (3, 11). Anti-CD28 monoclonal antibodies (mAbs) can prevent this induced unresponsiveness (4).

Another ligand for B7 is CTLA-4, a cell surface protein homologous to CD28, which is expressed on activated T cells (12, 13). CTLA4Ig, a soluble form of CTLA-4 can inhibit a variety of immunological responses (12, 14–16). We have shown that blocking the CD28 signaling pathway with CTLA4Ig blocks allo- and xenoantigen responses *in vitro*, prevents xenogeneic transplant rejection, and induces long-term, antigen-specific unresponsiveness *in vivo* (15).

In this study, we demonstrate the existence of an additional CTLA-4 ligand, B7-2, that provides a costimulatory signal to T cells. B7-2 binds to CTLA4Ig, is constitutively expressed on dendritic cells, and is rapidly up-regulated on activated B cells. Finally, B7-2 appears to play an important functional role in stimulating primary T-cell responses *in vitro*.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Mice. C57BL/6, DBA/2J, and BALB/cJ mice were obtained from The Jackson Laboratories and housed in a specific pathogen-free animal barrier facility at the University of Chicago.

Antibodies and Flow Cytometric Analysis (FCM). Cells (10^5) were incubated with 2.4G2 (a rat anti-mouse Fc receptor mAb) for 15 min in FACS buffer (0.1% bovine serum albumin/0.01% sodium azide) prior to staining to prevent antibody binding to Fc receptors (17). The cells were then washed and preincubated with uncoupled antibodies where indicated for 30 min at 4°C prior to adding fluorochrome-coupled reagents: human (h) CTLA4Ig (a gift from Peter Linsley, Bristol-Myers Squibb), 16-10A1 (a hamster anti-murine B7 mAb), and 1G10 (a rat IgG2a anti-murine B7 mAb) (18). They were incubated for 45 min to 1 hr and were then developed with phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates). B cells were detected with fluorescein isothiocyanate (FITC)-coupled B220 (RA3-6B2; PharMingen) while dendritic cells were stained with N418 (a hamster anti-murine CD11c mAb) and developed by a FITC-coupled anti-hamster immunoglobulin (Jackson ImmunolResearch). hCTLA4Ig and control immunoglobulin (control Ig) (Repligen) used in *in vitro* assays were prepared as described (19, 20). One- and two-color FCMs were performed with a FACScan flow cytometer (Becton Dickinson). Logarithmically amplified fluorescence data were collected on 10^4 viable cells as determined by forward and right angle light scatter intensity.

T-Cell Clones and Tumors. $B7^+$ CHO cells were generated as described (5). The P815 tumor line, transfected with murine B7 (P815-B7), were a gift from Lewis Lanier (DNAX). EL-4 cells (American Type Culture Collection) were cotransfected with MHC class II ($I\text{-}A^d$) cDNA and murine B7 cDNA and maintained in Dulbecco's modified Eagle's medium supplemented with fetal calf serum and G418 (21).

The Th1 clone PGL2, provided by Frank Fitch (University of Chicago), is specific for ovalbumin-(323–339) and is $I\text{-}A^d$ restricted. Clones were maintained as described (22).

Splenic Dendritic Cell Purification. Dendritic cell-enriched populations were prepared as low buoyant density, transiently adherent cells from mouse spleen cell suspensions (23). The dendritic cells collected from the plates were further enriched over a 50%/65% Percoll gradient and analyzed for purity by FCM analysis using N418, which has previously been shown to preferentially recognize dendritic cells purified in this manner (23).

Abbreviations: TCR, T-cell receptor; MHC, major histocompatibility complex; mAb, monoclonal antibody; FCM, flow cytometric analysis; FITC, fluorescein isothiocyanate; h, human; MLR, mixed lymphocyte response; APC, antigen presenting cell; LPS, lipopolysaccharide.

¶To whom reprint requests should be addressed.

T-Cell Proliferation Assays. Tumor cell transfectants were treated with mitomycin C (Sigma) for 30–45 min and BALB/c spleen stimulators were irradiated at 2000 rads (1 rad = 0.01 Gy) to prevent cell proliferation; 1×10^5 (transfectants) or 2.5×10^5 (irradiated spleen) stimulator cells, preincubated with blocking or control antibodies for 15 min, were added to individual wells of a 96-well microtiter plate. PGL2 T-cell clones, which had been rested 7 days and treated with anti-I-A^d mAb (M5/114; American Type Culture Collection) and complement (Accurate Chemical), were then added at a density of 5×10^4 cells per well in the presence or absence of antigenic peptide (DOT) (24). The 96-well plates were incubated at 37°C for 48 hr, pulsed with 1 μCi of [^{3}H]thymidine per well (1 Ci = 37 GBq), and harvested 12–16 hr later.

Whole spleen (5×10^5) or enriched dendritic cells (5×10^4) from C57BL/6 mice were irradiated (2000 rads) and incubated with blocking antibodies for 15 min prior to the addition of responders. DBA/2J lymph node responder cells (6×10^5) were added to mixed lymphocyte cultures and incubated at 37°C for 4 days, pulsed with 1 μCi of [^{3}H]thymidine per well for the last 12–16 hr, and harvested. Counts are represented as mean cpm of triplicate wells. SEMs were <10%.

PCR Analysis. RNA purification, cDNA, and PCR amplification were carried out as described with slight modifications (25). RNA was isolated by using guanidinium isothiocyanate lysing buffer with subsequent CsCl gradient purification as described. cDNA was then prepared by reverse transcription with Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL). The PCR was performed using *Taq* polymerase (Boehringer Mannheim) and a 5′ oligonucleotide primer of B7 (TGCTGTCTGTCATTGCTGGGAAACT) paired with a 3′ oligonucleotide primer of B7 (CCCAGGTGAAGTCCTCTGACACGTG). The B7 primers span an intron; therefore, no product will be produced after PCR of DNA (data not shown), excluding the possibility that exogenous B7 DNA was detected by PCR. Denaturation, annealing, and extension reactions were carried out at 94°C for 15 s, 56°C for 15 s, and 72°C for 30 s, respectively, for 45 cycles. In each experiment, the relative amount of cDNA was analyzed with a pair of β-actin primers: a 5′ oligonucleotide primer (TGGAATCCTGTGGCATCCATGAAAC) and a 3′ oligonucleotide primer (TAAAACGCAGCTCAGTAACAGTCCG). Each gel was photographed with negative film (Polaroid).

RESULTS

Differential Inhibition of an Allogeneic Mixed Lymphocyte Response (MLR) by Anti-B7 mAbs vs. hCTLA4Ig. Previous studies have shown that CTLA4Ig profoundly inhibits allogeneic and xenogeneic T-cell responses *in vivo* (15, 16). However, in preliminary experiments, we observed that anti-B7 mAbs were much less effective in suppressing islet transplant rejection (15) (D.J.L. and J.A.B., unpublished observations). Therefore, hCTLA4Ig and the anti-B7 mAbs 16-10A1 and 1G10 (data not shown) were examined for their ability to inhibit an *in vitro* allogeneic MLR. Only hCTLA4Ig was effective at inhibiting the allogeneic MLR using whole spleen cells as stimulators (Fig. 1).

Since dendritic cells are purported to be the predominant splenic subset responsible for initiating primary allogeneic T-cell responses (26), we examined the ability of hCTLA4Ig and anti-B7 mAb (16-10A1) to block an allogeneic MLR stimulated by highly enriched (40–90% N418$^+$) dendritic cells. Ten times less dendritic cells induced a 3 times greater proliferative response than splenic stimulator cells (Fig. 1). This proliferative response was effectively inhibited by hCTLA4Ig (>75%) but not by the anti-B7 mAbs (<10%). These observations raised questions with regard to the role of

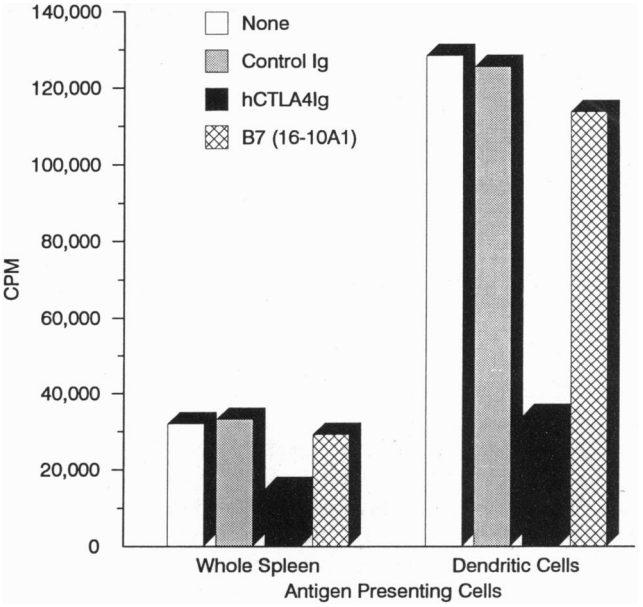

FIG. 1. hCTLA4Ig, but not anti-B7 mAbs, significantly inhibits a primary allogeneic MLR. DBA/2J lymph node responder cells were incubated with either whole C57BL/6 spleen or enriched C57BL/6 dendritic cells (70% pure) in the presence of the indicated blocking reagent (100 μg/ml). Open bars, response in the absence of blocking antibodies. Results are representative of 10 experiments.

B7 as a major cell adhesion/costimulation pathway in a primary allogeneic MLR.

The Anti-B7 Antibodies Have an Equivalent or Higher Avidity for B7 Than Does hCTLA4Ig. One possible trivial explanation for the ability of CTLA4Ig, but not anti-B7 mAbs, to block a primary MLR is that CTLA4Ig binds to B7 with a higher avidity or to a different epitope than does the anti-B7 mAb. As shown in Fig. 2, all three reagents were able to bind to the murine B7-transfected CHO cells (B7-tf CHO cells), and this binding was not inhibited by control Ig (dotted lines). By comparison, both of the anti-B7 mAbs (1G10 and 16-10A1) inhibited the binding of hCTLA4Ig biotin to the B7-tf CHO cells with equal or greater effectiveness than hCTLA4Ig (Fig. 2 *Left*, ■ and ▲ vs. ●). The 1G10 antibody blocked the binding of hCTLA4Ig biotin (Fig. 2 *Left*) and 1G10 FITC (Fig. 2 *Right*) to B7 at similar concentrations as hCTLA4Ig (● vs. ▲), whereas the 16-10A1 mAb (■) completely blocked hCTLA4Ig, 1G10, and 16-10A1 (Fig. 2 *Middle*) staining at 100-fold or lower concentrations than the amount of hCTLA4Ig needed for equivalent blocking of B7-specific binding by the various reagents. These data demonstrate that the anti-B7 mAbs have equivalent, if not greater, avidity for B7 than does hCTLA4Ig and that these reagents bind to overlapping epitopes on the B7 molecule.

Anti-B7 mAb and hCTLA4Ig Inhibit Proliferation of a Th1 Clone to Antigen-Pulsed B7 Transfectants but Not Spleen Cells. To formally demonstrate that anti-B7 mAb could block the functional interaction between B7 and CD28, we compared the ability of anti-B7 mAbs and CTLA4Ig to inhibit the potent proliferation of an ovalbumin-specific Th1 clone, PGL2, to either antigen-pulsed EL-4 cells expressing both I-A^d and B7 (ELAD-B7) or I-A^d-expressing BALB/c spleen cells (Fig. 3). While both hCTLA4Ig and the anti-B7 mAbs (16-10A1 and 1G10; data not shown) effectively inhibited T-cell proliferation to the ELAD-B7 cells, only hCTLA4Ig inhibited the proliferative response of PGL2 to antigen-pulsed spleen cells. In fact, the anti-B7 mAb (16-10A1) inhibited the ELAD-B7-mediated PGL2 stimulation substantially better at lower concentrations than either hCTLA4Ig or 1G10, most likely reflecting the apparent higher avidity of this anti-B7 mAb for

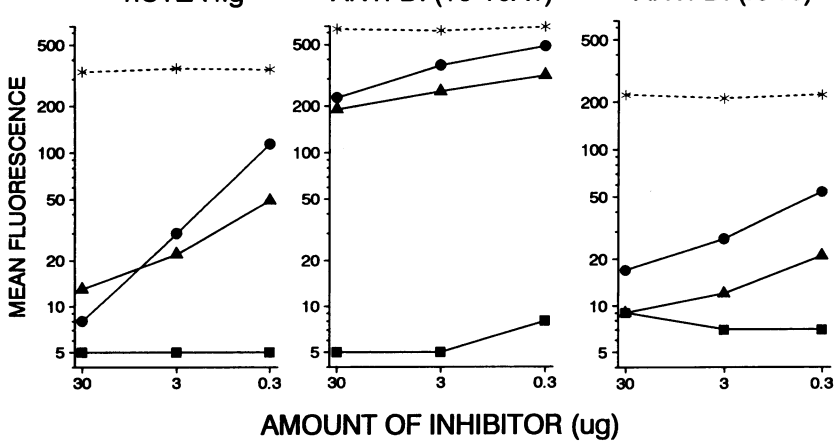

FIG. 2. Anti-B7 antibodies have equal or higher avidity for B7 than does hCTLA4Ig. B7-tf CHO cells were incubated with the indicated concentration of unlabeled blocking reagents: hCTLA4Ig (●), anti-B7 mAb 16-10A1 (■), or anti-B7 mAb 1G10 (▲). Cells were then stained with saturating amounts of biotin-coupled hCTLA4Ig (*Left*), biotin-coupled anti-B7 mAB 16-10A1 (*Middle*), or FITC-coupled anti-B7 mAb 1G10 (*Right*). In control experiments, untransfected CHO cells did not react with any of the three reagents (data not shown). Dotted line (asterisks) in each panel represents mean fluorescence of cells stained with the various reagents in the presence of control Ig. Background staining in the absence of fluorochrome-labeled reagents (<10) is subtracted from the values depicted.

B7. Thus, although all three reagents bind to the functionally relevant epitope on the B7 molecule, only hCTLA4Ig was able to inhibit the proliferative response to splenic antigen presenting cells (APCs). These data suggest that splenic APCs express an alternative ligand for CTLA-4 that can functionally provide costimulatory activity to both primary T cells and to established T-cell clones.

Additional Ligand for CTLA-4 Is Expressed on Activated Splenic B Cells and Dendritic Cells. Since the blocking studies on the B7-tf CHO cells showed that the anti-B7 antibodies could *completely* inhibit hCTLA4Ig staining of B7, we used this assay to examine whether specific ligands for hCTLA4Ig might exist on enriched dendritic cells. Dendritic cells stained with both hCTLA4Ig and anti-B7 mAb, confirming that these cells expressed B7 (Fig. 4 *I* and *J*) (7). However, hCTLA4Ig staining was not blocked by unlabeled anti-B7 mAb (16-10A1) (Fig. 4L) at a concentration that completely blocked CTLA4Ig binding to the B7-tf CHO cells (Fig. 2). These results suggest that, in addition to B7, another ligand(s) for hCTLA4Ig is also highly expressed on dendritic cells.

Although no more than 3% of freshly isolated splenocytes stained with either hCTLA4Ig or anti-B7 mAb (Fig. 4 *A–D*), stimulation of spleen cells with LPS for 3 days reproducibly resulted in the up-regulation of B7 on ≈50% of the splenic B cells (Fig. 4*F*). In contrast, >70% of the LPS-activated B cells (in some experiments, >90% of activated B cells) reacted with the hCTLA4Ig (Fig. 4*E*). Thus, hCTLA4Ig ligand binding is up-regulated on activated B cells that do not bind anti-B7 mAbs. In fact, unlabeled anti-B7 mAb, 16-10A1, or 1G10 (data not shown) only minimally inhibited CTLA4Ig staining of LPS-activated B cells even on B7$^+$ cells (Fig. 4 *E* vs. *G* and *H*). It should be emphasized that both of the

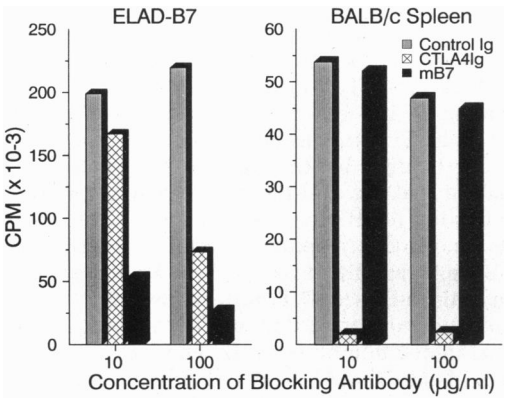

FIG. 3. Only hCTLA4Ig inhibits T-cell proliferation to antigen-pulsed whole spleen, while both hCTLA4Ig and anti-B7 mAbs block T-cell proliferation to antigen-pulsed B7 transfectant cells. The Th1 clone PGL2 was incubated with ELAD-B7 cells or irradiated BALB/c spleen cells pulsed with a tryptic digest of ovalbumin (DOT) (25 μg/ml). The proliferation of PGL2 to ELAD-B7 or BALB/c spleen cells and antigen without additional blocking antibodies was 229,252 and 59,958 cpm, respectively. No proliferation was observed when nontransfected EL-4 cells were used and an anti-hamster control antibody did not inhibit proliferation (data not shown). Results are representative of three experiments.

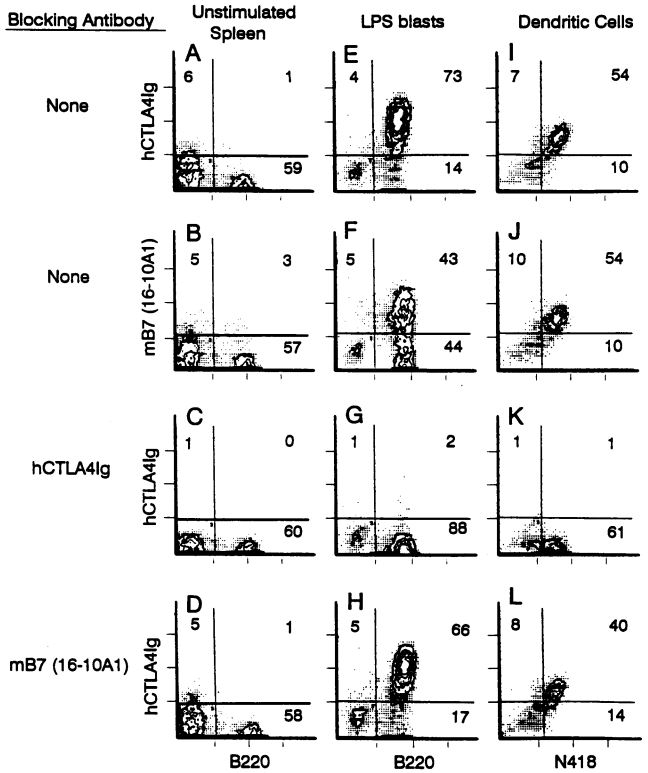

FIG. 4. An additional ligand for CTLA-4 is expressed on activated splenic B cells and dendritic cells. Splenic dendritic cells were isolated as described. Spleen cells were stimulated with lipopolysaccharide (10 μg/ml) for 3 days and then stained for B7 and B7-2 expression as described, with 30 μg of the indicated blocking antibodies used in these experiments. Percentage positive cells are indicated in each quadrant. In repeated experiments, a small number of B cells reacted preferentially with anti-B7 mAbs but less well with hCTLA4Ig (*A* vs. *B*). This binding was blocked by anti-B7 mAbs but not control Ig (data not shown). The amount of biotin coupled to the 16-10A1 mAb was substantially greater than to hCTLA4Ig. Thus, the relative fluorescence intensity of these two reagents cannot be compared.

unlabeled anti-B7 mAbs blocked the binding of FITC or biotin-coupled anti-B7 mAbs to the activated B cells and dendritic cells (data not shown). These results suggested that LPS activation of B cells up-regulated an additional ligand for hCTLA4Ig in addition to B7 on most B cells and on a population of activated B cells that were $B7^-$. We refer to this ligand as B7-2.

Differential Regulation of B7-2 and B7 Expression on Activated Splenic B Cells. B7 is maximally expressed on LPS-activated B cells after 48–72 hr (8). Since a costimulator molecule believed to influence primary T-cell responses might be expected to be expressed early after B-cell engagement, we compared the time course of expression of B7-2 and B7 after LPS activation. As expected, B7 was induced on splenic B cells stimulated with LPS, with maximal levels of expression observed by day 3. This was shown by staining with both of the anti-B7 mAbs (Fig. 5*A*; data not shown). In contrast, hCTLA4Ig binding was observed as early as 6 hr after LPS stimulation of splenic B cells. The CTLA4Ig reactivity was not blocked by inhibitory amounts of unlabeled anti-B7 mAbs, confirming that CTLA4Ig was binding to B7-2 and not B7. Expression of B7-2 was maximal by 24 hr and remained highly expressed until day 4.

We next examined whether B7-2 and B7 were up-regulated on murine T cells after activation with a potent T-cell mitogen, Con A. No hCTLA4Ig binding was observed on the activated T cells (data not shown). In contrast, hCTLA4Ig binding to B cells was observed as early as 6 hr after Con A activation (Fig. 5*A*). However, unlike LPS activation, no B7 expression was evident throughout the entire time course as judged by staining with the two different anti-B7 mAbs. In addition, B7-2 was induced by Con A on purified B cells, and this did not require the presence of T cells or T-cell-derived lymphokines (data not shown). While the data support the existence of another ligand for CTLA-4, we cannot eliminate the possibility that it is an alternative form of B7 that only binds to hCTLA4Ig. To address this possibility we performed extensive reverse transcriptase PCR analyses of B cells activated for various lengths of time with either Con A or LPS. As shown by Fig. 5*B*, only the B cells stimulated with LPS expressed significant amounts of B7 mRNA. The barely detectable levels of B7 mRNA in Con A-activated cells was due to either a small percentage of activated $B7^+$ B cells resident in the animals (Fig. 4) or dendritic cell contamination in the B-cell preparations, since equivalent levels of B7 mRNA were found on unstimulated cells (data not shown). Together these results indicate that not only does B7-2 expression occur prior to and, in some cases, independent of B7 expression, but independent signaling events may control the regulation of these molecules.

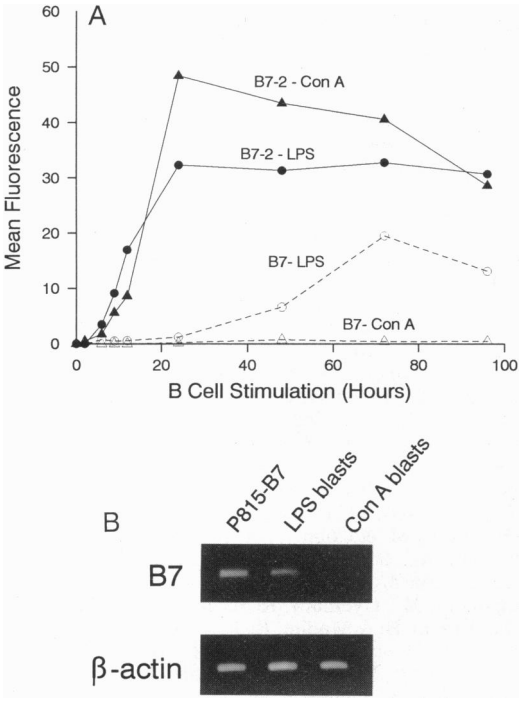

FIG. 5. Differential regulation of the B7-2 and B7 expression on activated splenic B cells. (*A*) Spleen cells from C57BL/6 mice were incubated with LPS (10 μg/ml) (circles) or Con A (2.5 μg/ml) (triangles) for the indicated time. B7-2 (solid lines) was measured as the net amount of mean fluorescence intensity (arbitrary units) observed with hCTLA4Ig biotin in the presence of uncoupled anti-B7 mAb (16-10A1) (background mean fluorescence intensity, <10). B7 expression was measured by staining with anti-B7 mAb (16-10A1) (dotted lines). All staining reagents and FACScan settings were kept constant so that the mean fluorescence intensity could be compared between time points. Incubation of the cells in medium for the duration of the time course did not up-regulate either B7 or B7-2 (data not shown). (*B*) B7 mRNA expression was analyzed by performing reverse transcriptase PCR analysis of the cDNA from purified B cells stimulated with either LPS (10 μg/ml) for 72 hr or Con A (5 μg/ml) for 24 hr. The expected size of the B7 PCR product is 458 bases. The relative amount of B7 cDNA was compared to a control PCR using β-actin-specific primers with an expected size of 345 bases.

DISCUSSION

The regulation of T-cell activation is quite complex, including not only antigen-specific recognition by the TCR, but also a cascade of adhesion and costimulatory events. These events are directed by various receptor–ligand pairs found on T cells and APCs, which have been shown to increase cellular adhesion between the T cell and APC and/or to provide necessary signals to costimulate both T and B cells (27). Another characteristic of these cell surface molecule interactions is that many of the receptors can bind to more than one ligand. This may reflect the ability of the various receptor–ligand interactions to provide signals that differentially regulate T- and B-cell responses based on tissue distribution or other variables.

In this study, we establish the existence of a second ligand for CTLA-4 (B7-2) and its functional relevance in the initiation of T-cell proliferation. B7-2 is expressed on splenic dendritic cells and is rapidly up-regulated on activated B cells. Murine B7-2 is required either alone or in conjunction with B7 to initiate T-cell proliferation to "natural" APCs, such as dendritic cells and whole spleen cells. Recently, it was demonstrated that activated keratinocytes express a B7-like molecule (BB-1) that interacts with CD28 (28). Since CTLA4Ig has been shown to bind to B7, BB1, and now B7-2, it is possible that B7-2 represents multiple gene products that are induced at different times or by different activation conditions. Until the gene(s) is cloned or antibodies are produced against these products this possibility cannot be dismissed.

Interestingly, the events controlling the regulation of B7 and B7-2 are distinct. While LPS-stimulated B cells expressed B7 only after 48 hr, B7-2 expression could be detected as early as 6 hr. In addition, Con A stimulation of splenic B cells resulted in the up-regulation of B7-2 but not B7, suggesting that although Con A does not induce B-cell proliferation, crosslinking of some cell surface protein must selectively signal the B cell to up-regulate B7-2. Both the CD40/gp39 and the TCR/class II MHC interactions have been shown to induce B7 expression on B cells (18, 29). Although these signaling events may also cause B7-2 expression, another Con A binding ligand (perhaps cell surface immunoglobulin) might selectively up-regulate B7-2 expression. Finally, a potential role of T-cell activation in inducing

B7-2 expression also was evident in these studies, since an increased expression of B7-2 on B cells activated by Con A in the presence of T cells was observed (data not shown). This augmented expression may be due to lymphokines released by stimulated T cells or, alternatively, T-cell activation may alter a receptor, such as LFA-1 (30) or CD40 ligand (29), to increase adhesion or signaling between the T and B cells.

In summary, these findings emphasize the complexities involved in T-cell activation and raise the question as to how all these receptor–ligand pairs interact to control immune responses. Recent studies have shown that both CD28 and CTLA-4 bind to B7 (12). Therefore, B7-2 is also likely to interact with both T-cell ligands. One possibility is that B7-2 is rapidly up-regulated after B-cell activation, perhaps with antigen via the immunoglobulin receptor, to constitute a relevant functional interaction molecule for CD28, which is constitutively expressed on naive T cells (31). This receptor–ligand interaction would provide the necessary costimulatory signals for T-cell activation, resulting in the up-regulation of CTLA-4 on the activated T cells 2–3 days later (13) (T. Walunas, D.J.L., and J.A.B., unpublished data). Concurrently, B cells up-regulate B7 levels via signaling through MHC class II, CD40, and perhaps lymphokines secreted by the activated T cells (18, 29). Since CTLA-4 has a higher affinity for B7 than does CD28, B7 might preferentially interact with CTLA-4 on the activated T cells (12). The receptor–ligand interaction between CTLA-4 and B7 could deliver positive signals to the T cell to maintain an ongoing response or signal the activated B cells to initiate immunoglobulin secretion, or, alternatively, the interaction may send negative signals to down-regulate T- or B-cell functions. In fact, results from our laboratory, have shown that while hCTLA4Ig and anti-BB1 mAb have profound inhibitory effects in both transplant and autoimmune models (15), anti-B7 antibody therapy either has no effect on immune responses *in vivo* or, in the autoimmune model, exacerbates the disease process (D.J.L., K. Herold, and J.A.B., unpublished data). These results support the possibility that B7 may deliver a negative signal when CTLA-4 is expressed on the activated T cell and that by blocking this signal an ongoing immune response cannot be down-regulated.

It is known that costimulatory signals need to be delivered to the T cell within hours of TCR engagement in order to prevent anergy induction. Yet, B7 is not expressed on B cells and macrophages until days after their activation. Our studies show that the up-regulation of B7-2 occurs within 6 hr of activation, which would allow it to provide the necessary signals to prevent anergy induction (32). These kinetics and its functional importance in initiating an immune response suggest that B7-2 may be playing an important role not only in stimulating T cells but it may also be acting as an important costimulatory molecule responsible for the prevention of anergy induction. It will be important in future studies to determine how these molecules interact with each other and whether the quality or quantity of the signals delivered by each molecule differs under various circumstances.

We thank Drs. A. Sperling, M.-L. Alegre, M. Naujokas, and R. Sciammas for their critical review and helpful comments throughout these studies; D. Decker for her assistance with the PCR analysis; and Dr. Peter Linsley for providing hCTLA4Ig. J.A.B. is recipient of an American Cancer Society faculty award and D.J.L. is recipient of a National Institutes of Health Medical Scientist Training Program Fellowship. This work was supported by U.S. Public Health Service Grants AI29531 and CA14599 to J.A.B. and AI42857 to J.M.

1. Schwartz, R. H. (1990) *Science* **248,** 1349–1356.
2. Schwartz, R. H., Mueller, D. L., Jenkins, M. K. & Quill, H. (1989) *Cold Spring Harbor Symp. Quant. Biol.* **54,** 605–610.
3. Jenkins, M. K., Taylor, P. S., Norton, S. D. & Urdahl, K. B. (1991) *J. Immunol.* **147,** 2461–2466.
4. Harding, F. A., McArthur, J. G., Gross, J. A., Raulet, D. H. & Allison, J. P. (1992) *Nature (London)* **356,** 607–610.
5. Linsley, P. S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N. K. & Ledbetter, J. A. (1991) *J. Exp. Med.* **173,** 721–730.
6. Reiser, H., Freeman, G. J., Razi-Wolf, Z., Gimmi, C. D., Benacerraf, B. & Nadler, L. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 271–275.
7. Larsen, C. P., Ritchie, S. C., Pearson, T. C., Linsley, P. S. & Lowry, R. P. (1992) *J. Exp. Med.* **176,** 1215–1220.
8. Razi-Wolf, Z., Freeman, G. J., Galvin, F., Benacerraf, B., Nadler, L. H. & Reiser, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4210–4214.
9. Freeman, G. J., Gray, G. S., Gimmi, C. D., Lombard, D. B., Zhou, L. J., White, M., Fingeroth, J. D., Gribben, J. G. & Nadler, L. M. (1991) *J. Exp. Med.* **174,** 625–631.
10. Linsley, P. S., Clark, E. A. & Ledbetter, J. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5031–5035.
11. Damle, N. K., Klussman, K., Linsley, P. S. & Aruffo, A. (1992) *J. Immunol.* **148,** 1985–1992.
12. Linsley, P. S., Brady, W., Urnes, M., Grosmaire, L. S., Damle, N. K. & Ledbetter, J. A. (1991) *J. Exp. Med.* **174,** 561–564.
13. Linsley, P. S., Greene, J. L., Tan, P., Bradshaw, J., Ledbetter, J. A., Anasetti, C. & Damle, N. K. (1992) *J. Exp. Med.* **176,** 1595–1604.
14. Linsley, P. S., Wallace, P. M., Johnson, J., Gibson, M. G., Greene, J. L., Ledbetter, J. A., Singh, C. & Tepper, M. A. (1992) *Science* **257,** 792–795.
15. Lenschow, D. J., Zeng, Y., Thistlethwaite, J. R., Montag, A., Brady, W., Gibson, M. G., Linsley, P. S. & Bluestone, J. A. (1992) *Science* **257,** 789–792.
16. Turka, L. A., Linsley, P. S., Lin, H., Brady, W., Leiden, J. M., Wei, R., Gibson, M. L., Zhen, X., Myrdal, S., Gordon, D., Bailey, T., Bolling, S. F. & Thompson, C. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11102–11105.
17. Unkeless, J. C. (1979) *J. Exp. Med.* **150,** 580–596.
18. Nabavi, N., Freeman, G. J., Gault, A., Godfry, D., Nadler, L. M. & Glimcher, L. H. (1992) *Nature (London)* **360,** 266–268.
19. Lindsten, T., Lee, K. P., Harris, E. S., Petryniak, B., Craighead, N., Reynolds, P. J., Lombard, D. B., Freeman, G. J., Nadler, L. M., Gray, G. S., Thompson, C. B. & June, C. H. (1993) *J. Immunol.* **151,** 3489–3499.
20. Boussiotis, V., Freeman, G., Gray, G., Gribben, J. & Nadler, L. M. (1993) *J. Exp. Med.*, in press.
21. Miller, J. & Germain, R. N. (1986) *J. Exp. Med.* **164,** 1478–1489.
22. Gajewski, T. F., Pinnas, M., Wong, T. & Fitch, F. W. (1991) *J. Immunol.* **146,** 1750–1758.
23. Steinman, R. M. & Cohn, Z. A. (1979) *J. Exp. Med.* **149,** 1–16.
24. Shimonkevitz, R. J., Kappler, J., Marrack, P. & Grey, H. (1983) *J. Exp. Med.* **158,** 303–316.
25. Yamamura, M., Uyemura, K., Dians, R., Weinberg, K., Rea, T. H., Bloom, B. & Modlin, R. L. (1991) *Science* **254,** 277–279.
26. Romani, N., Koide, S., Crowley, M., Witmer-Pack, M., Livingstone, A. M., Fathman, C. G., Inaba, K. & Steinman, R. M. (1989) *J. Exp. Med.* **169,** 1169–1178.
27. Van Seventer, G. A., Shimizu, Y. & Shaw, S. (1991) *Curr. Opin. Immunol.* **3,** 294–303.
28. Nickoloff, B., Mitra, R. S., Lee, K., Turka, L. A., Green, J., Thompson, C. & Shimizu, Y. (1993) *Am. J. Pathol.* **142,** 1029–1040.
29. Ranheim, E. A. & Kipps, T. J. (1993) *J. Exp. Med.* **177,** 925–935.
30. Hibbs, M. L., Xu, H., Stacker, S. A. & Springer, T. A. (1991) *Science* **251,** 1611–1613.
31. Gross, J. A., Callas, E. & Allison, J. P. (1992) *J. Immunol.* **149,** 380–388.
32. Go, C. & Miller, J. (1992) *J. Exp. Med.* **175,** 1327–1336.