

## Functional Expression of the Costimulatory Molecule, B7/BB1, on Murine Dendritic Cell Populations

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### Summary

Whereas dendritic cells (DC) are known to be potent activators of T cells both in vitro and in vivo, the critical costimulatory molecules expressed on DC are not well characterized. Using immunocytochemical and molecular techniques we find that splenic DC express B7/BB1, the counter-receptor for CD28. Moreover, expression of B7/BB1 is upregulated on epidermal Langerhans cells (LC) during their functional maturation into potent T cell stimulators. In blocking experiments, we find that participation of B7/BB1 is required for optimal proliferation of unprimed, allogeneic T cells in DC-driven, primary mixed leukocyte reactions. These data demonstrate that the regulated expression of B7/BB1 on DC may be important in the initiation of a primary T cell response.

Dendritic cells (DC), and to a lesser extent B cell blasts, are the principal activators of resting T cells in both murine and human in vitro T cell proliferation assays (1, 2). Moreover, DC are the most potent initiators of primary T cell responses in vivo (3). The identity of the critical costimulatory signals for resting T cell activation possessed by DC and B blasts has remained elusive. Failure of DC to synthesize IL-1 dispelled the notion that this cytokine is the critical "second signal" (4). Likewise, although intercellular adhesion molecule 1 (ICAM-1)/LFA-1 and LFA-3/CD2 are important adhesion and costimulatory molecules, their distribution on a variety of cell types indicates that their expression alone would not account for the unique ability of DC and B blasts to activate resting T cells (5).

Recent attention has focused on CD28, which is expressed on most CD4<sup>+</sup> and ~50% of CD8<sup>+</sup> T cells, as a putative receptor of the second signal required for T cell activation (for review see reference 6). A homologous molecule, CTLA4, has been cloned from cytotoxic T cells, suggesting that a member of the CD28 family of accessory molecules may be expressed on all T cells (7). Signals delivered via CD28 enhance cytokine gene transcription (8), stabilize cytokine message (9), and are required for optimal proliferation and cytokine release by CD4<sup>+</sup> T cells and Th1 cell lines (10, 11).

A ligand for CD28, B7/BB1 (12), has been identified on IFN- $\gamma$ -treated monocytes (13) and activated, but not resting B cells (14). There is evidence indicating that its participation may be important in the activation of resting T cells in the MLR (15). In light of their stimulatory properties, it seems likely that DC express B7/BB1 or a similar CD28 counter-receptor. Moreover, knowledge that DC exist in dis-

tinct phenotypic and functional forms suggests that the expression of B7/BB1 may be regulated in DC. Resident Langerhans cells (LC), the DC of skin, can process antigens, but are poor activators of resting T cells (16, 17). However, in bulk epidermal cell cultures, under the regulation of the cytokine GM-CSF, LC mature into extremely potent T cell stimulators (18). Although the molecular mechanisms responsible for Langerhans cell maturation are unknown, the characteristics of B7/BB1 suggest that it may play an important role in this process.

### Materials and Methods

**Mice.** Male C57BL/10, BALB/c, C3H/He, B10.BR, and DBA/2 mice (7–12-wk-old) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) or The Jackson Laboratory (Bar Harbor, ME).

**Antibodies.** The following were used as primary antibodies for flow cytometric analysis: TIB 145, rat anti-mouse P50 (B cell restricted; American Type Culture Collection, Rockville, MD); N418 (19), hamster anti-mouse CD11c (DC restricted in mice); biotinylated B21-2, rat anti-mouse Ia<sup>b,d</sup> (kind gift from Justin A. Roake and Jonathan M. Austyn, University of Oxford); and anti-CD4-PE (Becton Dickinson & Co., Mountain View, CA).

The CTLA4-Ig fusion protein, contains the extracellular portion of human CTLA4 and an Ig C $\gamma$  chain, and the B7-Ig fusion protein is comprised of the extracellular portion of human B7 and the same Ig C $\gamma$  chain (7). CTLA4-Ig binds to human, rat, and mouse B7/BB1 in the concentration range used in these studies (3–30  $\mu$ g/ml). B7-Ig has very low avidity for human and murine CD28, and shows very little binding in the same concentration range ([7] and Peter Linsley, unpublished observations).

Second-stage reagents and antibodies used for these experiments

included: FITC-conjugated goat anti-human IgG (Sigma Chemical Co., St. Louis, MO), biotin and FITC conjugates of rabbit anti-hamster IgG (Accurate Chemical & Scientific Corp., Westbury, NY), biotin and FITC conjugates of goat anti-rat-IgG (Sigma Chemical Co.), and streptavidin-PE (Becton Dickinson & Co.).

**Splenic Dendritic Cells.** DC-enriched populations were prepared as low-buoyant density, transiently-adherent cells from mouse spleen cell suspensions (20). DC within these preparations were labeled with the hamster anti-mouse DC mAb, N418, followed by biotinylated rabbit anti-hamster IgG (Accurate Chemical & Scientific Corp.), and finally streptavidin-PE (Becton Dickinson & Co.).

**Epidermal Cell Suspensions.** Epidermal sheets were prepared using  $2.5 \times$  trypsin/EDTA (Sigma Chemical Co.) essentially as described (18). The cells released from the sheets were passed through 100  $\mu$ m nylon filters, pelleted, and counted. The resultant epidermal cell suspension (2–5% Langerhans cells) were either used immediately (fresh EC, ECf) or cultured (cultured EC, ECc) at a density of  $1-2 \times 10^6$ /ml in complete media (RPMI 1640 containing 5% FCS, 2 mM glutamine, and antibiotics,  $2.5 \times 10^{-5}$  M 2-ME) for 3 d.

**T Cells.** Primary T cell-enriched populations were prepared from unfractionated spleen cell suspensions as nylon wool-nonadherent cells followed by red cell lysis in Tris-buffered ammonium chloride. EDS-1 is an alloreactive (B10.BR anti-C57BL/10) CD4<sup>+</sup> T cell line derived in our laboratory.

**LPS-activated Splenocytes.** Spleen cell suspensions were depleted of red cells using Tris-buffered ammonium chloride. Splenocytes were cultured at  $10^6$ /ml in complete media containing 10  $\mu$ g/ml LPS (*Escherichia coli* B5:055, Sigma Chemical Co.) for 72 h.

**Flow Cytometric Analysis.** Cell populations were labeled as follows: splenic DC were labeled with the hamster anti-mouse DC mAb, N418, followed by biotinylated rat anti-hamster-IgG blocked with 5% normal mouse serum and then streptavidin-PE. LC within epidermal cell suspensions were labeled with biotinylated B21-2 (anti-Ia<sup>b,d</sup>) followed by streptavidin-PE, the only Ia<sup>+</sup> cells within epidermal cell suspensions being LC (16). B cells were identified with the rat mAb, TIB 145, a B cell-restricted marker, followed by FITC conjugate goat anti-rat IgG blocked with 5% normal mouse serum. Negative controls were either rat anti-horseradish peroxidase or no primary.

B7/BB1 expression on the various cell populations was assessed using the CTLA4-Ig fusion protein at 3–5  $\mu$ g/ml followed by FITC-conjugated goat anti-human IgG, blocked with 5% normal mouse serum. Negative controls included the B7-Ig fusion protein (5  $\mu$ g/ml), human IgG (10  $\mu$ g/ml) (Sigma Chemical Co.), or no primary reagent with equivalent results.

Fluorescence profiles were generated using a FACScan<sup>®</sup> flow cytometer and Consort 30 software (Becton Dickinson & Co.). Sterile cell sorts were performed on a FACStar<sup>®</sup> flow cytometer (Becton Dickinson & Co.).

**T Cell Proliferation Assays.** In allogeneic MLR, graded doses of irradiated (2,000 rad, <sup>137</sup>Cs) stimulator cells were added to  $4 \times 10^5$  allogeneic nylon wool-passed responder cells in a final volume of 150  $\mu$ l in 96-well round-bottomed microtest plates. Proliferation was measured by adding 1  $\mu$ Ci of [<sup>3</sup>H]methyl-thymidine (Amersham Corp., Arlington Heights, IL)/well after 72 h. The cells were harvested 12–16 h later and counted on a BetaPlate Counter (LKB Instruments, Inc., Gaithersburg, MD). Results are the means of triplicate cultures.

**PCR.** mRNA was prepared from  $3-8 \times 10^6$  LPS-activated splenocytes, or  $2 \times 10^5$  sorted DC using the Microfast Tract System (Invitrogen, San Diego, CA), which employs SDS lysis followed by isolation of mRNA on oligo dT cellulose. First-strand

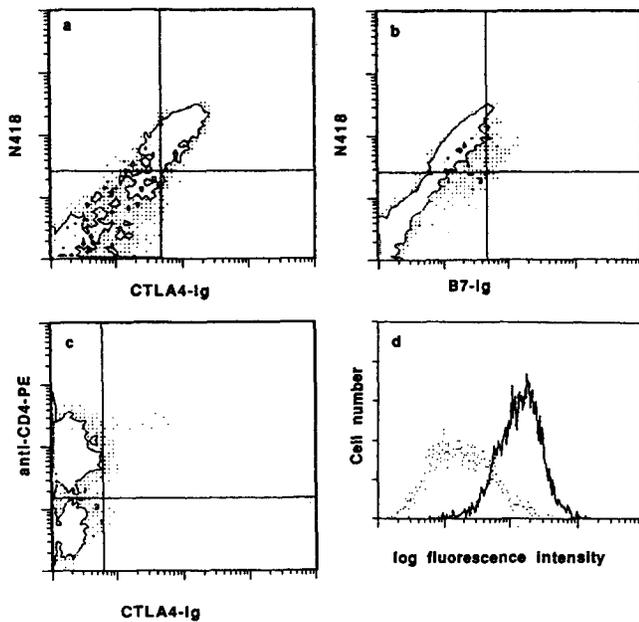
cDNA synthesis was performed using avian myeloblastosis reverse transcriptase (Invitrogen) using an oligo dT primer. PCR was carried out in a 50- $\mu$ l volume using 5  $\mu$ l of cDNA, 5  $\mu$ l  $10 \times$  PCR reaction buffer (Amersham Corp.), 1.5 U Hot tub DNA polymerase (Amersham Corp.), 0.5 mM dNTPs, 50 pmol of forward and reverse oligonucleotide primers (5'ATGGCTTGCAATGTCAG3'-sense, nucleotides 249–266 of the murine B7/BB1 cDNA sequence and 5'CTAAAGGAAGACGGTCT3'-antisense, nucleotides 1153–1169), synthesized at the Microchemical Facility of the Winship Cancer Center, Emory University (21). The coding sequence of murine B7/BB1 gene is composed of at least five exons and spans  $\sim 20$  kb (21). Since the oligonucleotide primers used in these experiments come from different exons (i.e., include the start and stop codons, respectively), amplification of contaminating genomic DNA would yield distinctly different sized product (i.e.,  $\sim 20$  kb) than the  $\sim 920$  bp product seen in our reactions. Reactions were carried out for 40 cycles of 95°C for 1 min, 50°C for 45 s, and 72°C for 1 min in a thermocycler (Perkin-Elmer Cetus Corp., Norwalk, CT).

The PCR products were analyzed by agarose gel electrophoresis, transferred to nylon membranes (Tropix, Inc., Orem, UT), then immobilized by UV fixation. The blots were probed with a biotinylated internal B7/BB1 oligonucleotide (5' AGTTGTCCATCAAAGCTGAC 3', nucleotides 661–680, synthesized and labeled by the Microchemical Facility of the Winship Cancer Center). The biotinylated probe and marker tract were visualized using the Southern Light chemiluminescence system (Tropix, Inc.).

## Results and Discussion

**Splenic Dendritic Cells Express the Costimulatory Molecule, B7/BB1.** Our initial experiments were designed to study the expression of the B7/BB1 costimulatory molecule on murine cell populations with particular emphasis on determining whether B7 is expressed on mature immunostimulatory dendritic cells. As there are no mAbs that recognize murine B7/BB1, we used the CTLA4-Ig fusion protein as the first stage reagent for FACS<sup>®</sup> analysis. As noted in the introduction, CTLA4 is a natural ligand for B7/BB1. Using the PCR, Linsley et al. (7) have developed a fusion protein that contains the binding region of CTLA4 and the C region of the human IgG H chain. Their studies with this fusion protein have demonstrated its use as a probe that binds to human, rat, and mouse B7/BB1 ([7], and Peter Linsley, unpublished observations).

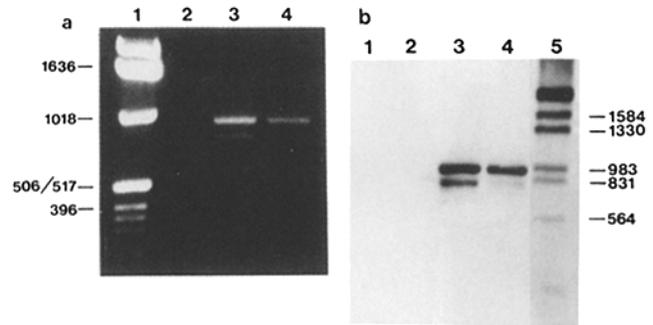
DC-enriched low density, transiently-adherent spleen cells were analyzed for expression of B7 using two-color immunofluorescence on the flow cytometer. For comparison, LPS-activated B cell blasts and a CD4<sup>+</sup> T cell line, EDS-1 were also evaluated. As expected, the T cell line failed to express a CTLA4 counter-receptor (Fig. 1 c), whereas LPS-activated B cells strongly expressed a ligand for CTLA4-Ig (Fig. 1 d). In addition, we found that N418 positive immunostimulatory DC also strongly bound the CTLA4-Ig fusion protein, but not the B7-Ig fusion protein (Fig. 1, a and b) or human IgG (data not shown). In contrast, the N418-negative fraction, which is composed of T cells, B cells, and macrophages, failed to label with CTLA4-Ig. These binding studies provided initial evidence that DC and LPS-activated



**Figure 1.** Immunostimulatory dendritic cells and LPS-activated B cells express B7/BB1. Dendritic cell-enriched populations were prepared from C57BL/10 spleens and labeled with N418 (a hamster anti-mouse DC mAb). B7 expression was assessed on the DC (PE positive) using the CTLA4-Ig fusion protein as a first-stage reagent followed by FITC-conjugated goat anti-human IgG. For comparison, LPS-activated B cell blasts and a CD4-positive alloreactive T cell line (EDS-1) were also studied. (a) Splenic dendritic cells labeled with both N418 and CTLA4-Ig. (b) Splenic DC failed to label with the control B7-Ig fusion protein. In contrast, the CD4-positive alloreactive T cell line failed to bind CTLA4-Ig (c). LPS-activated B cell blasts which uniformly expressed TIB 145 (a B cell-restricted marker; not shown) also expressed B7/BB1 (d) when analyzed by single-color flow cytometry. (· · ·) Negative control, (—) CTLA4-Ig. Two-color data (a-c) depict log fluorescence intensity on both x and y axes. Single-color histogram (d) depicts log fluorescence intensity on the x axis, and relative cell number on the y axis. (Similar results have been obtained in three experiments).

B cell blasts, but not T cells, express B7/BB1 or a novel counter-receptor/ligand for CTLA4.

In an attempt to distinguish between these possibilities, we used the PCR to detect B7/BB1 transcripts. Highly purified DC populations were isolated by fluorescence activated cell sorting N418 positive cells from low-buoyant density, transiently-adherent spleen cell suspensions. mRNA from these cells and from LPS-activated splenocytes was prepared, followed by first-strand cDNA synthesized on an oligo dT primer. cDNA from the populations was then amplified using specific B7/BB1 oligonucleotide primers (21). PCR products were analyzed by agarose gel electrophoresis. Using this approach we detected similar sized PCR products (~920 and ~800 bp) from both LPS-activated blasts and dendritic cell preparations, but not from reactions in which no cDNA template was added (Fig. 2 a). Specificity of the amplification was confirmed by hybridizing Southern blots of the gels with a biotinylated B7/BB1 oligonucleotide probe which is contained within the DNA amplified by the PCR primers. Both the ~920 and the ~800 bp bands hybridized with the B7



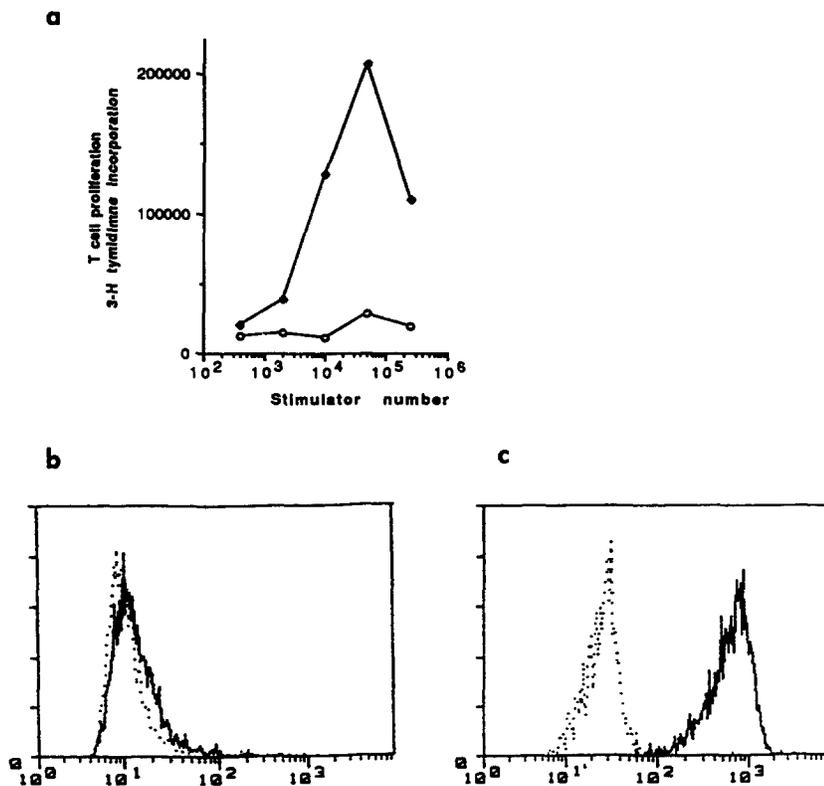
**Figure 2.** Dendritic cells and LPS-activated B cells express B7/BB1 mRNA. mRNA was prepared from  $2 \times 10^5$  sorted N418-positive DC or  $5 \times 10^6$  LPS blasts followed by cDNA synthesis (20  $\mu$ l final volume). 5  $\mu$ l of cDNA was then amplified using specific B7/BB1 oligo nucleotide primers from different exons of the coding sequence. PCR was carried out for 40 cycles and analyzed on ethidium bromide-stained 1.5% agarose gels. (a) Similar ~920 and ~800 bp bands were seen in PCR reactions of cDNA from LPS blasts (lane 3) and dendritic cells (lane 4), but not in PCR samples with no cDNA added (lane 2). (b) Southern blots of the gels were then probed with an internal biotinylated B7 oligonucleotide probe confirming the specificity of the amplification. Lanes 1, DNA molecular weight standards (1 kb ladder, Gibco BRL, Gaithersburg, MD); 2, no cDNA; 3, LPS blasts; 4, dendritic cells; and 5, biotinylated DNA M.W. standards ( $\lambda$  DNA EcoRI, HindIII; Sigma Chemical Co.).

oligo probe (Fig. 2 b) but not with a  $\beta$ -actin probe (data not shown). B7 RNA transcripts of 10, 4.2, 2.9, and 1.7 kb and protein products with molecular masses ranging from 45 to 66 kD have been reported (14, 21).

Together, the two-color flow cytometric data showing cell surface expression of a CTLA4 ligand, and the PCR data demonstrating presence of B7/BB1 mRNA in splenic DC provide convincing evidence that splenic DC express B7/BB1. However, we cannot formally exclude the possibility that DC may also express an additional novel CTLA4 ligand.

*B7/BB1 Expression Is Upregulated on Epidermal Langerhans Cells during Their Functional Maturation In Vitro.* As noted earlier, freshly isolated LC (LCf) display numerous phenotypic differences from DC isolated from lymphoid tissues. However, in culture, LC (LCc) "mature" into cells that are virtually phenotypically and functionally indistinguishable from lymphoid DC (16, 18). LCf are relatively weak stimulators of resting T cells, whereas LCc are extremely potent T cell activators (16). Similarly, we find that freshly prepared bulk epidermal cell suspensions (ECf) are weak stimulators of allogeneic T cells, whereas cultured bulk EC (ECc) induce vigorous proliferation of allogeneic T cells (Fig. 3 a). Previous studies have demonstrated that these differences in stimulatory capacity of ECf and ECc are a reflection of LC maturation, and that these phenotypic and functional changes are induced by the cytokine GM-CSF (18).

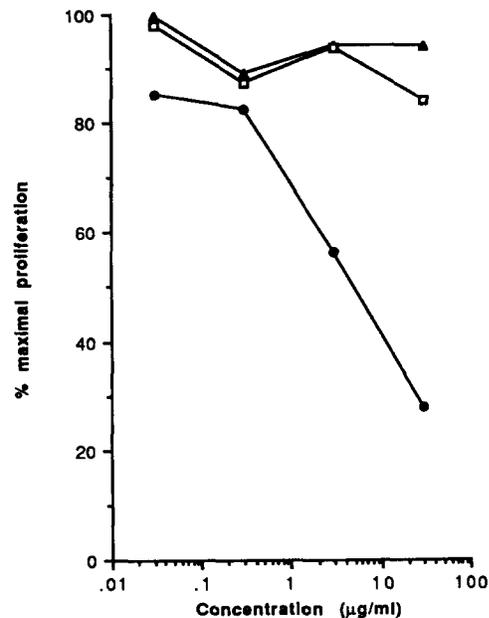
We sought to test the hypothesis that differential expression of the B7/BB1 costimulatory molecule by mature LC may be responsible for the observed differences in immunostimulatory function of freshly isolated and cultured Langerhans cells. Consistent with this notion, we have found that expression of B7 is strongly upregulated on epidermal



**Figure 3.** Epidermal LC upregulate expression of B7 during their functional maturation in culture. (a) Graded doses of irradiated freshly isolated (open circles) or cultured (closed diamonds) C57BL/10 epidermal cells were added to  $4 \times 10^5$  nylon wool passed B10.BR T cells in round-bottomed 96-well microtest plates. T cell proliferation was measured as  $[^3\text{H}]$ thymidine incorporation after 72 h in culture. Results are means of triplicate cultures. As reported by Freeman et al. (21), we found that cultured EC are many times more potent T cell stimulators than freshly isolated EC. B7 expression was assessed on freshly isolated and cultured LC (3 d) using two-color flow cytometry. (b) Freshly isolated LC do not express detectable levels of B7 (CTLA4 binding) relative to the negative control. In contrast, (c) cultured LC strongly express B7. (· · ·) Negative control; (—) CTLA4-Ig. Negative controls have included B7-Ig, human IgG, and no primary with identical results. Single-color histograms (b and c) depict log fluorescence intensity on the x axis and relative cell number on the y axis. Similar results have been obtained in three experiments. Failure to label freshly isolated LC is not an artifact of the isolation procedure as the level of CTLA4-Ig binding on the  $\text{Ia}^+$  LC was only minimally altered after reexposure of bulk EC to  $2.5 \times$  trypsin/EDTA for 10 min at  $37^\circ\text{C}$ . CTLA4-Ig mean channel fluorescence-negative control on LCc is 975, and after reexposure to trypsin, 882. Similar results were obtained for splenic DC and LPS-activated B cell blasts.

LC during their maturation into potent T cell stimulators. LC within epidermal cell suspensions were labeled with biotinylated B21-2 followed by streptavidin-PE. B7/BB1 expression was again assessed using CTLA4-Ig followed by FITC-conjugated goat anti-human IgG. The cell populations were then analyzed using two-color flow cytometry. Using this approach, LCf did not express detectable levels of B7. In marked contrast, LCc were found to express high levels of B7 (Fig. 3, b and c). These results could not be explained by recovery of a trypsin-sensitive marker since reexposure of cultured EC to trypsin/EDTA did not significantly affect expression of B7/BB1 on the LCc (see Fig. 3 legend). The Ia-negative fraction, which is composed of keratinocytes and  $\gamma/\delta$  T cells failed to label with CTLA4-Ig in fresh and cultured preparations (data not shown).

*Participation of B7/BB1 Is Required for Activation of Resting Allogeneic T Cells by Splenic Dendritic Cells, and Antigen-driven Proliferation of Th1 Cells.* To explore the functional role of B7/BB1 on DC during the initiation of T cell responses, we tested the ability of CTLA4-Ig to inhibit dendritic cell-driven primary allogeneic MLR. We find that CTLA4-Ig produces a dose-dependent inhibition of the allo MLR, whereas neither human IgG nor the B7-Ig altered DC-induced T cell proliferation (Fig. 4). Although this data demonstrates that B7 is a critical accessory molecule in DC-driven primary T cell responses, it remains unclear whether CTLA4-Ig inhibits the MLR exclusively by blocking signal transduction through CD28, or if blocking B7/BB1-CD28-mediated adhesion also contributes to the inhibition of DC-induced T cell proliferation. Our preliminary observations suggest that the initial



**Figure 4.** CTLA4-Ig inhibits dendritic cell-induced T cell proliferation in an allogeneic MLR. Unprimed C3H/He nylon wool-passed T cells ( $4 \times 10^5$ /well) were stimulated with  $10^5$  C57BL/10 dendritic cells in the presence of increasing doses of CTLA4-Ig, B7-Ig, or human IgG. T cell proliferation was measured as  $[^3\text{H}]$ thymidine incorporation after 3 d and is expressed as the percentage of incorporation in untreated cultures. Results shown are the means of triplicate cultures. Addition of CTLA4-Ig (closed circles) resulted in a dose-dependent inhibition of proliferation whereas neither B7-Ig (closed triangles), which has very low avidity for mouse CD28, nor human IgG (open squares) affected the proliferative response. Similar results have been obtained in three experiments.

development of DC-T cell clusters is not inhibited by CTLA4-Ig, but confirmation of these observations will require the development of semiquantitative cluster assays.

As noted above, signals delivered via CD28 augment release of IL-2 and IFN- $\gamma$ , but not IL-4, suggesting that the effect of CD28 signaling may differ in Th1 and Th2. We have also observed that CTLA4-Ig inhibits antigen-specific proliferative responses of a Th1 clone, but has much less effect on the specific proliferation of a Th2 clone (our unpublished observations). Analysis of additional Th1 and Th2 clones will be required to assess the significance of these preliminary observations.

In summary, we find that: (a) mature splenic dendritic cells express B7/BB1 (during the preparation of this manuscript we have become aware that another group has described similar expression [22]); (b) epidermal Langerhans cells upregulate expression of B7 during their functional maturation into potent T cell stimulators; and (c) B7/BB1 expression by den-

dritic cells is of critical importance in the activation of unprimed allogeneic T cells.

The distribution, functional, and migratory properties of dendritic cells are ideally suited to their role as specialized initiators of T cell-mediated immune responses. Our data bring together the observations that DC upregulate immunostimulatory function during their migration from peripheral tissues into T cell regions of lymphoid tissues (23, 24) and provide evidence that expression of B7/BB1 plays a critical role in this process. Further studies on the cytokines that regulate the expression and function of B7/BB1 on dendritic cells and other APC may provide additional insights into the mechanisms involved in the initiation and amplification of T cell-mediated immune responses, and lead to the development of new strategies to up- or downregulate this molecule and thus augment (e.g., vaccination), interrupt (e.g., allograft rejection), or alter the character (Th2 vs. Th1) of nascent or ongoing immune responses.

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We thank Bristol-Myers Squibb for providing the CTLA4-Ig and B7-Ig fusion proteins. We also thank Linda Stempora for assistance with cell sorting; Drs. Wright Caughman and Peter Jensen for helpful discussions; and Dr. John D. Whelchel for his advice and encouragement throughout these studies.

C.P. Larsen is the recipient of an American College of Surgeons Fellowship Award. This work was supported in part by National Institutes of Health grant 5R01 AI-30732-02.

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Received for publication 29 May 1992 and in revised form 14 July 1992.

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