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# The B7/BB1 Antigen Provides One of Several Costimulatory Signals for the Activation of CD4<sup>+</sup> T Lymphocytes by Human Blood Dendritic Cells In Vitro

James W. Young,\*\* Lydia Koulova,<sup>§</sup> Steve A. Soergel,\* Edward A. Clark,<sup>†</sup> Ralph M. Steinman,\* and Bo Dupont<sup>§</sup>

\*Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021; †Bone Marrow Transplantation Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, Cornell University Medical College, New York 10021;

§Human Immunogenetics Laboratory, Sloan-Kettering Institute for Cancer Research, New York 10021; and \*\*Department of Microbiology, University of Washington, Seattle, Washington 98195

## Abstract

T cells respond to peptide antigen in association with MHC products on antigen-presenting cells (APCs). A number of accessory or costimulatory molecules have been identified that also contribute to T cell activation. Several of the known accessory molecules are expressed by freshly isolated dendritic cells, a distinctive leukocyte that is the most potent APC for the initiation of primary T cell responses. These include ICAM-1 (CD54), LFA-3 (CD58), and class I and II MHC products. Dendritic cells also constitutively express the accessory ligand for CD28, B7/BB1, which has not been previously identified on circulating leukocytes freshly isolated from peripheral blood. Dendritic cell expression of both B7/BB1 and ICAM-1 (CD54) increases after binding to allogeneic T cells. Individual mAbs against several of the respective accessory T cell receptors, e.g., anti-CD2, anti-CD4, anti-CD11a, and anti-CD28, inhibit T cell proliferation in the dendritic cell-stimulated allogeneic mixed leukocyte reaction (MLR) by 40–70%. Combinations of these mAbs are synergistic in achieving near total inhibition. Other T cell-reactive mAbs, e.g., anti-CD5 and anti-CD45, are not inhibitory. Lymphokine secretion and blast transformation are similarly reduced when active accessory ligand–receptor interactions are blocked in the dendritic cell-stimulated allogeneic MLR. Dendritic cells are unusual in their comparably higher expression of accessory ligands, among which B7/BB1 can now be included. These are pertinent to the efficiency with which dendritic cells in small numbers elicit strong primary T cell proliferative and effector responses. (*J. Clin. Invest.* 1992. 90:229–237.) **Key words:** dendritic cells • B7/BB1 • CD28, T cell activation • antigen-presenting cells • costimulation

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The current address of L. Koulova is Dept. of Membrane Research and Biophysics, The Weizmann Institute of Science, 76100 Rehovot, Israel.

Address correspondence to Dr. James W. Young, Laboratory of Cellular Physiology and Immunology, Box 280, The Rockefeller University, 1230 York Ave., New York, NY 10021.

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## Introduction

Antigen-presenting cells (APCs)<sup>1</sup> express cell surface molecules that are known to enhance T cell responses to peptide-MHC complexes (1–3). These costimulatory or accessory molecules pair with specific receptors on the T cell. Several of these molecular couples have been characterized: LFA-3 (CD58) with CD2 (4, 5), ICAM-1 (CD54) with LFA-1 (CD11a) (6), B7/BB1 with CD28 (7–10) and/or CTLA-4 (11), CD72 with CD5 (12), and class I or II MHC with CD8 (13) or CD4 (14), respectively. However, these accessory ligands have not always been identified on circulating leukocytes, and their roles have usually been evaluated using expanded T cell clones already activated by antigen and heterogeneous feeder cells.

Prior work has established that dendritic cells, which represent a trace but distinctive leukocyte subset, are the most potent APC for the induction of primary, antigen-specific T cell responses in vitro and in situ (15). Among the known accessory couples, the interaction between B7/BB1 and CD28 is the only one that appears to stimulate T cell proliferation by a direct effect on IL-2 production (16–19). We therefore investigated whether dendritic cells expressed the accessory molecule B7/BB1, in addition to their known high expression of ICAM-1 (CD54), LFA-3 (CD58), and MHC class I and II products (20–22). We report that all are expressed by dendritic cells and that B7/BB1 and ICAM-1 (CD54) specifically increase after dendritic cell binding to primary CD4<sup>+</sup> lymphocytes. Using mAbs directed against the T cell receptors for these costimulatory molecules, we also demonstrate the functional roles of these accessory couples in the complete activation of primary T cells in the allogeneic mixed leukocyte reaction (MLR).

## Methods

### *Culture medium, serum, and buffers*

Cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10 mM HEPES (Sigma Chemical Co., St. Louis, MO), 1 mM glutamine (JRH Biosciences, Lenexa, KS),  $5 \times 10^{-5}$  M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY), penicillin (100 U/ml)-streptomycin (100 µg/ml) (JRH Biosciences), and 5 or 10% serum. FCS (JRH Biosciences) was used for short term culture of T cells, before stimulation in MLRs. Normal human serum (NHS), obtained from fasting, healthy, untransfused male donors was used to culture all non-T cell fractions and allogeneic MLRs. Only Ca<sup>2+</sup>- and

1. *Abbreviations used in this paper:* APCs, antigen-presenting cells; B-LCL, B-lymphoblastoid cell lines; ER<sup>-</sup>, erythrocyte-negative; ER<sup>+</sup>, erythrocyte-positive; MLR, mixed leukocyte reaction.

Mg<sup>2+</sup>-free saline buffers (PBS or HBSS) or bovine calf serum (BCS; JRH Biosciences) 5% vol/vol in RPMI were used for cell washes. All sera were heat-inactivated at 56°C for 30 min to deplete complement. All cell cultures were maintained at 37°C in humidified 7% CO<sub>2</sub>.

### Monoclonal antibodies

mAbs used in the functional *in vitro* assays and for the isolation of T lymphocyte subsets were: anti-HLA class II, DR-specific (L243, IgG2a; Xth International Histocompatibility Workshop), and monomorphic DR/DQ-specific (9.3F10, IgG2a, HB180; American Type Culture Collection [ATCC], Rockville, MD); anti-CD2, recognizing the LFA-3 (CD58) binding site (TS<sup>2</sup>/18, IgG1, HB 195; ATCC); anti-CD4 (Leu3a/IgG1; gift of Dr. Robert Evans, New York); anti-CD5 (6-2, IgG2a; B. Dupont); anti-CD8 (OKT8, IgG2a, CRL 8014; ATCC; SA 19-11, IgG1; gift of Dr. S. Y. Yang, New York); anti-CD11a (TS1/22, IgG1, HB 202; ATCC); anti-CD14 (3C10, IgG2b, TIB 228; ATCC; LeuM3, IgG1; Becton-Dickinson Microbiology Sys., Mountain View, CA); anti-CD16 (Leu11b, IgM; Becton-Dickinson Microbiology Sys.); anti-CD18 (IB4, IgG2a; gift of Dr. S. D. Wright, New York [23]); anti-CD25 (anti-TAC, IgG2a; gift of Dr. T. Waldmann, Bethesda, MD; 6G, IgG2a, gift of Dr. S. Y. Yang, New York); anti-CD28 (9.3, IgG2a; gift of Dr. Jeffrey Ledbetter, Seattle, WA); anti-CD45 (4B2, IgG2a, HB 196; ATCC); anti-CD45RA (4G10, IgG2a; R. M. Steinman); anti-CD45RO (UCHL-1, IgG2a; gift of Dr. P. C. L. Beverley, London, UK [24, 25]); anti-CD54 (LB2, IgG2a, FITC-conjugated; E. A. Clark); anti-CD56 (Leu19, IgG1; Becton-Dickinson Microbiology Sys.); anti-CD57 (Leu7, IgM; Becton Dickinson Microbiology Sys.); anti-CD58 (LFA-3, IgG1, HB 205; ATCC); anti-BB1 (anti-B cell activation antigen B7/BB1, IgM, both unconjugated and FITC-conjugated; E. A. Clark [26]). All murine anti-human mAbs used in the functional *in vitro* assays were purified as whole immunoglobulin from murine ascites or serum-free hybridoma supernatants and quantified at OD<sub>280</sub>. F(ab')<sub>2</sub> fragments were not used in the functional assays, as blood dendritic cells do not have detectable FcR (27). For isolation of lymphocyte subsets, either murine ascites or hybridoma supernatants were applied at previously determined effective concentrations. Cytofluorographic analyses of T lymphocyte subset purity and accessory ligand expression were performed on a FACScan® instrument (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Commercial mAbs for staining were purchased already conjugated to either FITC or PE; otherwise, FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse IgG + IgM (4353; TAGO, Inc., Burlingame, CA) was used as a second-step reagent for indirect staining. We FITC-conjugated anti-BB1 and LB2 (CD54) according to standard methods (28). Stained cells were analyzed by cytofluorography on a FACScan® instrument (Becton-Dickinson Immunocytometry Systems). Commercial murine IgG1/MOPC21 and IgM/TEPC 183 (Sigma Chemical Co.) were used as isotype-matched controls if comparable T cell-reactive mAbs were not available.

Because a human blood dendritic cell-specific mAb was not available, dendritic cells were analyzed cytofluorographically by gating cells with large forward scatter that did not stain with a panel of PE-conjugated cell-specific mAbs to B and T lymphocytes, monocytes/macrophages, and NK cells ([20]; see also Results, Fig. 1). Having excluded nondendritic cell PBMC from the analysis gate, dendritic cells were then analyzed for positive counterstaining by a FITC-conjugated mAb to the epitope of interest.

### PBMC and preparation of leukocyte subpopulations

PBMC and the mononuclear subpopulations of interest were prepared according to previously published procedures (20, 27, 29). In summary, hepatitis and HIV-seronegative PBMC were obtained from freshly drawn normal leukocyte concentrates (Greater NY Blood Program, New York) by centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) at 1,000 g for 20 min at room temperature. PBMC were washed in PBS and rosetted with neuraminidase-treated (*Vibrio cholerae* neuraminidase; Calbiochem-Behring Corp., LaJolla,

CA) sheep erythrocytes (Cocalico Biologicals, Inc., Reamstown, PA). These were separated over Ficoll-Paque into erythrocyte-negative (Er<sup>-</sup>) and erythrocyte-positive (Er<sup>+</sup>) fractions.

*Purification of T lymphocytes and their subsets.* Er<sup>+</sup> lymphocytes were passed over nylon wool columns (18369; Polysciences, Inc., Warrington, PA), and CD4<sup>+</sup> T lymphocytes were purified by negative selection from the Er<sup>+</sup>, nylon wool nonadherent fraction. Cells opsonized by mAbs against CD8, CD14, CD16, CD25, CD56, CD57, and MHC class II loci were eliminated by (a) two rosettings with goat-anti-mouse Ig-coated magnetic beads [Dynabeads M-450; DYNAL Inc., Great Neck, NY], followed by Ab/C' (rabbit complement; Gibco Laboratories)-mediated lysis (30); or (b) two to three pannings over goat-anti-mouse IgG-coated (55459, anti-IgG Fc  $\gamma$ -chain specific; Cappel, Organon Teknika, Durham, NC) petri dishes (31, 32). Cytofluorography confirmed a resulting population of > 98% CD3<sup>+</sup>/CD4<sup>+</sup> T cells, contaminated by < 1% HLA-DR<sup>+</sup>, CD25<sup>+</sup>, CD16<sup>+</sup> cells. For some preliminary experiments we also separated the CD4<sup>+</sup> lymphocytes into CD4<sup>+</sup>/CD45RA<sup>-</sup> and CD4<sup>+</sup>/CD45RO<sup>-</sup> subsets by similar negative selection (30).

*Enrichment of dendritic cells (20, 27, 29).* After the Er<sup>-</sup> fraction had been cultured ~ 36 h in RPMI 10% NHS, the nonadherent cells were collected and panned twice over human Ig-coated (10 mg/ml; Cappel, Organon Teknika) petri dishes to deplete contaminant monocytes, which adhered via their Fc receptors (27). A final adherence step to tissue culture plastic was performed at 37°C for 30–45 min. This Er<sup>-</sup>FcR<sup>-</sup> plastic nonadherent population was resuspended, overlaid on 3 ml of 14.5 g% metrizamide [20, 29]; Metrizamide AG, 222010; Nycomed AS, Oslo, Norway), and centrifuged at 650 g for 10 min at room temperature. The resulting interface was collected and washed twice in decreasingly hypertonic medium (11.34 and 10.43 g% NaCl in RPMI 5% FCS, equivalent to 40 mM and 25 mM NaCl, respectively) before a final wash in isotonic medium (20). This interface contained blood dendritic cells at ~ 40–60% purity, easily identified by phase microscopy on a hemacytometer, the major contaminants being B lymphocytes by cytofluorographic analysis. The purity of the DC population could be improved to 80–90% by panning depletion of residual CD45RA<sup>+</sup> (4G10) B lymphocytes and CD14<sup>+</sup> (3C10) monocytes (20). The high density pellet from the metrizamide gradient consisted almost entirely of B lymphocytes and was discarded.

### Allogeneic MLR

T cells and dendritic cells were combined from random allogeneic leukocyte concentrates. Enriched dendritic cells were  $\gamma$ -irradiated (3,000 rad 137Cs) and added to T lymphocytes at the APC:T ratios indicated in the respective experiments, typically 1:25 or 1:30. MLRs were cultured in RPMI 10% NHS, either in 16 mm, 24-well flat-bottomed (1.5  $\times$  10<sup>6</sup> T cells/well; Costar Corp., Cambridge, MA) or 96-microwell flat-bottomed (1.5  $\times$  10<sup>5</sup> T cells/well; Corning Glass, Inc., Corning, NY) tissue culture plates.

Inhibitory effects of various mAbs were assayed in the allogeneic MLR. T cells (or APCs in the case of anti-BB1 and anti-class II MHC) were incubated directly with the mAb(s) of interest in flat-bottomed microwells for 30–60 min at 4°C with gentle shaking. The cells were not washed before addition of the opposite MLR party. The cells were then placed at 37°C for the duration of culture. In certain experiments in which mAbs were added at different time points during culture, the plate was placed at 4°C for 30–60 min after addition of the mAbs and then returned to 37°C.

MLR proliferative activity was measured by the incorporation of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/microwell, New England Nuclear, Boston, MA) over 8–12 h typically on d4  $\rightarrow$  5. Cells were harvested on glass fiber filters and counted (1205 Betaplate; Pharmacia LKB Biotechnology, Inc., Gaithersburg, MD). Responses have been reported as the mean counts per minute  $\pm$  standard deviation of triplicates, and percent inhibition by mAbs has been calculated with regard to isotype-matched controls.

### Assay of T cell growth factor in the MLR

Aliquots of MLR supernatants were harvested at 40–48 h and frozen until assayed. The growth of a CTLL2 line in the presence of MLR supernatants was compared with that of a standard curve using graded doses of rhIL-2 ([33]; Cetus Corp., Emeryville, CA). The amount of T cell growth factor in the MLR supernatants was then expressed in terms of IU rhIL-2/ml (1 Cetus Unit = 6 IU rhIL-2).

### Assay of blast transformation in the MLR

Cohort wells of allogeneic MLRs being assayed for proliferation were stained with 0.5% vol/vol propidium iodide (P4170; Sigma Chem. Co.) and monitored by cytofluorography (FACScan; Becton-Dickinson Immunocytometry Systems). Propidium iodide-(FL2) positive cells were nonviable and excluded from analysis. Propidium iodide-negative cells were gated on the basis of forward light scatter in order to calculate the percentage of blasts.

### Separation of alloreactive T cell-dendritic cell aggregates or clusters

Previous studies have shown that dendritic cells and responding T cells in the MLR cluster together to form discrete aggregates (31, 34, 35). After ~ 40 h of MLR culture, half the supernatant from each MLR in a 16-mm macrowell was removed and replaced with serum-free RPMI, resulting in 5% vol/vol NHS/RPMI. The cells were gently harvested, overlaid on 5 ml of 30% vol/vol FCS/RPMI, and placed on ice. Alloreactive T cells, which had clustered with allogeneic dendritic cells in the MLR, settled by gravity over ~ 45 min. Nonreactive T cells in the responder population that had not formed clusters remained at or near the interface. Clusters were collected from the lowest 2 ml, washed in Ca<sup>2+</sup> and Mg<sup>2+</sup>-free PBS, vigorously resuspended, and counted. Clusters were either analyzed by cytofluorography, as an enriched source of dendritic cells that had bound alloreactive T cells, or they were recultured at 2.5–5 × 10<sup>4</sup> cells/100 μl in triplicate round-bottomed microwells for assessment of proliferation.

MLR clusters could be sufficiently disrupted at 40 h to release adequate numbers of individual dendritic cells for cytofluorographic analysis. However, the reverse was not true for clustered T cells, some of which remained tightly bound to dendritic cells that invariably contaminated the T cell gate on cytofluorography. Therefore, separate analyses of lymphoblasts were undertaken using highly purified (≥ 99%) CD4<sup>+</sup>CD3<sup>+</sup>, APC-free lymphocytes stimulated for 2 d with concanavalin A ([Con A] 4 μg/ml), and PMA (5 ng/ml).

## Results

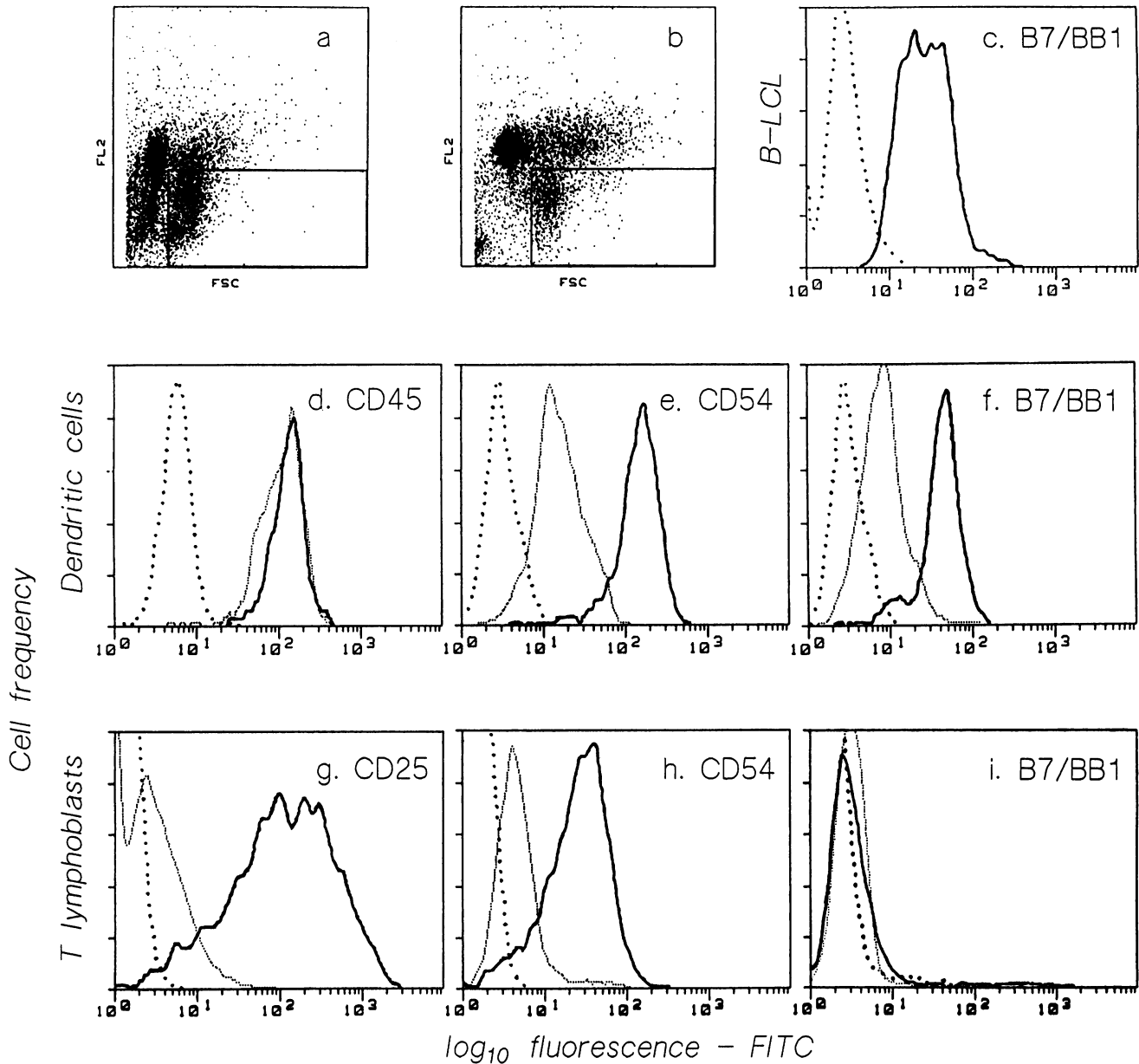
*Human blood dendritic cells express accessory ligands and increase their expression of B7/BB1 and ICAM-1 (CD54) after binding alloreactive T cells in the MLR.* Enriched populations of blood dendritic cells were analyzed by cytofluorography for their expression of the accessory ligands B7/BB1, ICAM-1 (CD54), LFA-3 (CD58), and class II MHC loci. Analyses were conducted both before and after antigen-specific binding or clustering with alloreactive CD4<sup>+</sup> T cells in the MLR. Primary populations of circulating blood dendritic cells consistently expressed detectable levels of B7/BB1 (Fig. 1 *f*), although the intensity of constitutive expression varied slightly between donors. Freshly enriched blood dendritic cells also expressed high amounts of ICAM-1 (CD54) (Fig. 1 *e*), as well as LFA-3 (CD58) and MHC products, as previously reported (20, not shown here). After Ag-specific binding with alloreactive CD4<sup>+</sup> T cells, however, dendritic cells upregulated their expression of both ICAM-1 (CD54) and B7/BB1 (Fig. 1, *e* and *f*). The increments in ICAM-1 (CD54) and B7/BB1 expression by clustered dendritic cells were specific, as we found no change in the ex-

pression of the leukocyte common antigen, CD45 (Fig. 1 *d*). LFA-3 (CD58) expression also did not change, and expression of MHC class II antigens DR and DQ increased variably (not shown). These findings were not altered by γ-irradiating (<sup>137</sup>Cs, 3,000 rad) the dendritic cells, increasing dendritic cell purity to 80–90% by panning depletion of residual CD45RA<sup>+</sup> and CD14<sup>+</sup> contaminants in the metrizamide interface, or clustering dendritic cells with bulk T cell responders in lieu of CD4<sup>+</sup> T cells in the MLR (not shown). We attempted to assess alterations in accessory ligand expression by dendritic cells cultured in the absence of allogeneic T cells after γ-irradiation. Purified γ-irradiated dendritic cells recultured alone for 40 h exhibited variable viability that unfortunately prevented reliable evaluation. When the cells did remain viable, only slight increases were noted in the expression of both B7/BB1 and ICAM-1 (CD54), but always less than after antigen-specific clustering with T cells in the allogeneic MLR.

Although T cells should have been excluded by the cytofluorographic gating of dendritic cells above, we evaluated whether T lymphoblasts themselves could express B7/BB1. We compared APC-free, Con A/phorbol myristate acetate (PMA)-stimulated T cells with unstimulated T cell controls for ICAM-1 (CD54) and B7/BB1 expression. We confirmed activation by the expression of p55IL-2R (CD25; Fig. 1 *g*). These CD25<sup>+</sup> T lymphoblasts increased their expression of the widely distributed ICAM-1 (CD54) marker as expected (Fig. 1 *h*). However, B7/BB1 was not expressed significantly above background by either the unstimulated controls or the Con A/PMA-elicited T lymphoblasts (Fig. 1 *i*). We therefore infer that T lymphocytes do not contribute to the increased B7/BB1 found on clustered dendritic cells in the allogeneic MLR.

*The accessory ligands expressed by blood dendritic cells are involved in stimulating primary T cell proliferative responses to alloantigen.* We first studied the function of the CD28:B7/BB1 couple, as blood dendritic cells constitute a primary leukocyte subpopulation expressing B7/BB1. The function of CD28 and B7/BB1 in allogeneic MLRs had previously only been assessed using B lymphoblastoid cell line stimulators (36). We therefore stimulated CD4<sup>+</sup> responder T cells with allogeneic dendritic cells in the presence of mAbs against B7/BB1 and its receptor CD28, singly and in combination. A representative experiment is shown in Fig. 2. Compared with isotype-matched controls, anti-CD28 consistently inhibited the primary allogeneic MLR by 40–70% (representative of 10 experiments). Anti-BB1 was typically less inhibitory than anti-CD28 (26–37% inhibition, cf. IgM control in this experiment). The combination of mAbs against BB1 and CD28, however, did not significantly increase the inhibition effected by either mAb alone, consistent with involvement of the same ligand:receptor couple in the dendritic cell T cell system. In contrast, the combination of anti-CD28 and anti-CD2 (TS2/18) was additive in its inhibitory effects, confirming interactions between more than one accessory ligand/receptor pair.

We then studied the function of other accessory ligands expressed by circulating blood dendritic cells (Table I). Intact mAbs against the T cell accessory molecules, CD2, CD4, CD11a, and CD28, were used to monitor inhibition of dendritic cell ligand binding and resultant T cell proliferation. The dose of mAb(s) was varied while the responder:stimulator ratio was held constant (Table I *A*), or vice versa (Table I *B*). Only bulk CD4<sup>+</sup> lymphocyte responses are illustrated, since prelimi-

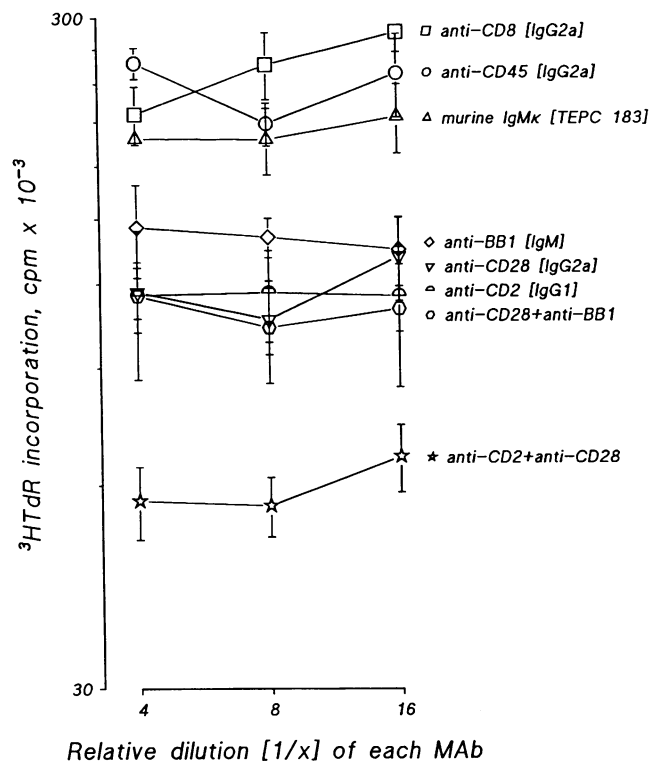


**Figure 1.** Cytofluorographic analysis of accessory ligand expression by human blood dendritic cells. Human blood dendritic cells were gated for cytofluorographic analysis based on large forward scatter and negative staining by a panel of PE-conjugated cell-specific mAbs to B and T lymphocytes, monocytes/macrophages, and NK cells (20): (a) immediately after enrichment; and (b) after resuspension of dendritic cells from dendritic cell-T cell clusters that had formed 40 h after initiation of the allogeneic MLR. This excluded nondendritic cell leukocytes from analysis when dendritic cells were assessed for positive counterstaining by a FITC-conjugated mAb to the accessory ligand of interest: (d, e, f) . . . . freshly enriched dendritic cells; — clustered dendritic cells; . . . . isotype-matched controls. B lymphoblastoid cell lines that constitutively express B7/BB1 served as positive controls: (c) — anti-B7/BB1; . . . . isotype-matched control. T lymphoblast expression of these ligands was also analyzed using highly purified, APC-free CD3<sup>+</sup>/CD4<sup>+</sup> T cells stimulated by Con A/PMA: (g, h, i) . . . . unstimulated T cells; — Con A/PMA-stimulated blasts; . . . . isotype-matched controls. T cell activation was confirmed by the expression of p55IL-2R/CD25: (g) unstimulated CD3<sup>+</sup>/CD4<sup>+</sup> = 2% CD25<sup>+</sup>, 96% CD25<sup>-</sup>; Con A/PMA-stimulated CD3<sup>+</sup>/CD4<sup>+</sup> blasts = 82% CD25<sup>+</sup>, 16% CD25<sup>-</sup>. Cytofluorographs are representative of six experiments. Dendritic cells used here were obtained from the Er<sup>-</sup>FcR<sup>-</sup>, plastic nonadherent, metrizamide interface fraction. Increased purity of the DC population to 80–90%, achieved by panning depletion of residual CD45RA<sup>+</sup> (4G10) and CD14<sup>+</sup> (3C10) cells in the metrizamide interface, did not alter the results shown.

nary experiments showed CD4<sup>+</sup>/CD45RA<sup>-</sup> and CD4<sup>+</sup>/CD45RO<sup>-</sup> subsets were indistinguishable in their inhibition by the various mAbs tested. We also compared T cells obtained by E-rosetting with those obtained by nonadherence to tissue culture plastic after 1 h incubation of PBMC, eluted from nylon wool columns in either case. There were no differences be-

tween T cells prepared by these two methods in terms of mean peak fluorescence of CD2 (TS2/18) surface expression at 40–48 h, or alloantigen responses in the MLR (six experiments; data not shown).

Single mAbs against T cell accessory molecules only partially inhibited the dendritic cell-stimulated allogeneic MLRs,



**Figure 2.** The costimulatory role of the B7/BB1:CD28 accessory ligand:receptor couple in the dendritic cell-stimulated allogeneic CD4<sup>+</sup> MLR.  $1.5 \times 10^5$  CD4<sup>+</sup> T cells were coated with anti-CD2 (TS2/18), -CD8 (OKT8), -CD28 (9.3), and/or -CD45 (4B2); and  $6 \times 10^3$  allogeneic dendritic cells were coated with anti-BB1, anti-HLA DR/DQ, or murine IgM $\kappa$  (TEPC 183). Opsonization was performed directly in flat-bottomed microwells for 30–60 min at 4°C with gentle shaking, after which the opposite party of allogeneic T cells or dendritic cells was added for a final responder:stimulator ratio of 25:1/microwell. The final concentrations of mAbs were 1/4, 1/8, and 1/16 dilutions of the following working concentrations: anti-CD2 50  $\mu$ g/ml, anti-CD8 50  $\mu$ g/ml, anti-CD28 128  $\mu$ g/ml, anti-CD45 90  $\mu$ g/ml, anti-BB1 50  $\mu$ g/ml, anti-HLA DR/DQ 72  $\mu$ g/ml, murine IgM $\kappa$  50  $\mu$ g/ml. MLRs were cultured in triplicate for each mAb dose and condition. Cells were pulsed for 8 h on d4  $\rightarrow$  5 of the MLR with 1  $\mu$ Ci<sup>3</sup>HTdR/microwell. Results shown are the mean counts per minute  $\pm$  standard deviation of triplicates and are representative of six experiments. Control MLR without mAb:  $243,376 \pm 16,985$ ; anti-HLA DR/DQ inhibited proliferation > 99% at all doses.

compared with isotype-matched controls. Anti-CD4 effected inhibition similar to the others, but is not illustrated. None of these mAbs blocked the initial binding of allogeneic dendritic cells and T cells by inverse phase microscopy inspection of the cultures (not shown). Combinations of these mAbs were additive in their inhibition of T cell proliferation. Saturating concentrations of paired mAbs blocked > 80%, with no substantial differences between groupings. Total inhibition of T cell proliferation (i.e.,  $\geq 97$ –98%) required saturating doses of at least three mAbs. Alternatively, by maintaining constant the total combined dose of two or more mAbs, there was a trend toward more complete inhibition as the amount of stimulatory alloantigen was decreased by adding fewer allogeneic dendritic cells. Addition of anti-CD4 did not further enhance the already maximal inhibition achieved by the combination of anti-CD2 + 11a + 28 (data not shown). Two other T cell-reactive mAbs against molecules involved in T cell signaling and activation,

CD5 and CD45 (37), were not inhibitory; resultant T cell proliferation varied less than  $\pm 10\%$  from control MLR cultures without mAb. We conclude that dendritic cells deliver several accessory signals that work in concert in an additive fashion. B7/BB1 can now be added to the other known dendritic cell costimulatory ligands, which together fully activate primary T cells.

*MAbs that inhibit proliferation similarly affect IL-2 release and blast transformation in the allogeneic MLR.* The amount of <sup>3</sup>HTdR incorporation on d4-5 is a distal measurement of accessory cell-T cell interactions that have occurred much earlier in the dendritic cell-stimulated MLR. We therefore evaluated whether two other parameters of T cell activation, IL-2 secretion and blast transformation, were concordant with the results obtained from <sup>3</sup>HTdR incorporation in parallel MLRs cultured under identical conditions. As shown in Fig. 3, these two independently measured parameters, IL-2 release by 48 h of culture and percent blast transformation on d4-5, were both highly correlated with actual T cell proliferation. We conclude that anti-T cell mAbs with specificity for accessory molecule receptors not only inhibit proliferation in the dendritic cell-stimulated allogeneic MLRs, but similarly decrease IL-2 release and actual blast transformation.

*Dendritic cells deliver important costimulatory signals early in the primary allogeneic MLR.* As dendritic cells are the most potent leukocyte for the initiation of T cell responses to alloantigen and mitogen (27), we hypothesized that their costimulatory signals were important for their accessory cell function early in the MLR. As illustrated in Fig. 4, addition of mAbs against T cell accessory receptor molecules from the outset of the MLR culture was needed to achieve significant inhibition. Equivalent inhibition was achieved at any dose of dendritic cell stimulators used (T:DC 30:1 illustrated; 10:1 and 100:1, not shown). Addition of mAbs at 24 or 48 h was less effective.

## Discussion

Primary antigen-specific T cell responses require peptide antigen presented in association with surface MHC products on APCs (1). Antigen-presentation alone is insufficient, however, to initiate effective T cell immunity (1, 32). The complete activation of T lymphocytes in a primary response therefore depends on the concerted delivery by APCs of several accessory or costimulatory signals in addition to Ag/MHC (1–3).

We present evidence here that human dendritic cells freshly isolated from peripheral blood express B7/BB1, the physiologic ligand for the CD28 T cell accessory molecule. Dendritic cells in blood (20) and skin (21, 22) also express high amounts of other accessory ligands, e.g., ICAM-1 (CD54), LFA-3 (CD58), and class II MHC products, particularly compared with other circulating leukocytes. B7/BB1 and ICAM-1 (CD54) expression by dendritic cells is dynamic, increasing after antigen-specific binding with histoincompatible T cells in the MLR. Other known accessory molecules, e.g., CD45, LFA-3 (CD58), and class II MHC, are fairly constant in their expression by blood dendritic cells.

B7/BB1 is constitutively expressed by B lymphoblastoid cell lines (B-LCL) (26, 36, 38, 39), as well as by  $\gamma$ -interferon (IFN)-stimulated macrophages (40). B7/BB1 is the accessory ligand for CD28 (7, 10) and/or CTLA-4 (11). Several experimental models have documented its costimulatory properties for T lymphocyte proliferation and lymphokine secretion, by

**Table I. Mean Percent Inhibition of Dendritic Cell-stimulated Allogeneic MLRs, by Monoclonal Antibodies Against T Cell Costimulatory Molecules**

A. Variable mAb dose [ $\mu\text{g/ml}$ ] per mAb or combination as indicated; Constant CD4 <sup>+</sup> T cell responder: allogeneic dendritic cell stimulator ratio of 30:1					
Monoclonal antibodies	mAb dose ( $\mu\text{g/ml}$ )	15	5	1.5	0.5
$\alpha\text{CD2}$ , TS2/18, IgG1		48	46	33	23
$\alpha\text{CD11a}$ , TS1/22, IgG1		57	44	27	5
$\alpha\text{CD28}$ , 9.3, IgG2a*		48	49	52	53
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$	Dose indicated	89	80	66	45
$\alpha\text{CD2}$ + $\alpha\text{CD28}$	per each mAb	85	82	78	61
$\alpha\text{CD11a}$ + $\alpha\text{CD28}$		85	78	68	54
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$ + $\alpha\text{CD28}^\ddagger$		97	94	85	74
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$	Dose indicated is	80	62	37	11
$\alpha\text{CD2}$ + $\alpha\text{CD28}$	combined Total	80	75	67	56
$\alpha\text{CD11a}$ + $\alpha\text{CD28}$		79	70	60	57
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$ + $\alpha\text{CD28}^\ddagger$		96	84	73	63

B. Constant mAb dose [ $15 \mu\text{g/ml}$ ] per mAb or combination as indicated; variable CD4 <sup>+</sup> T cell responder: allogeneic dendritic cell stimulator ratios				
Monoclonal antibodies	T:DC ratio	30:1	100:1	300:1
$\alpha\text{CD2}$ , TS2/18, IgG1		52	56	72
$\alpha\text{CD11a}$ , TS1/22, IgG1		66	68	77
$\alpha\text{CD28}$ , 9.3, IgG2a*		49	74	68
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$	15 $\mu\text{g/ml}$ per	94	96	97
$\alpha\text{CD2}$ + $\alpha\text{CD28}$	each mAb	86	86	85
$\alpha\text{CD11a}$ + $\alpha\text{CD28}$		90	94	92
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$ + $\alpha\text{CD28}^\ddagger$		98	100	98
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$	15 $\mu\text{g/ml}$ total,	82	90	96
$\alpha\text{CD2}$ + $\alpha\text{CD28}$	all mAbs combined	80	80	82
$\alpha\text{CD11a}$ + $\alpha\text{CD28}$		83	92	98
$\alpha\text{CD2}$ + $\alpha\text{CD11a}$ + $\alpha\text{CD28}^\ddagger$		98	99	99

*Table I.*  $1.5 \times 10^5$  CD4<sup>+</sup> T cells were coated directly in triplicate flat-bottomed microwells with the indicated mAbs for 30 min at 4°C, after which  $\gamma$ -irradiated allogeneic dendritic cells were added. Either the ratio of T cell responders to allogeneic dendritic cell stimulators [T:DC ratio] or the dose of mAb(s) was held constant, while the other parameter was varied. <sup>3</sup>HTdR incorporation was measured over 8–12 h on days 4–5 of the allogeneic MLR. Percent inhibition was calculated by comparing proliferation in the experimental wells with that in replicate control wells where T cells had been coated with isotype-matched controls, singly and in combination. Mean percent inhibition was calculated from four separate allogeneic combinations in *A* and from two separate allogeneic combinations in *B*. At a T:DC ratio of 30:1, the <sup>3</sup>HTdR incorporation [counts per minute  $\pm$  standard deviation] of quadruplicate control MLRs without mAb ranged from 62,709  $\pm$  15,373 to 216,028  $\pm$  29,669 amongst the different allogeneic pairings. \* Addition of  $\alpha\text{BB1}$  never increased the inhibition effected by  $\alpha\text{CD28}$  alone (see also Fig. 2). <sup>‡</sup> Addition of  $\alpha\text{CD4}$  to this combination did not increase the already maximal inhibition (see also Fig. 2).

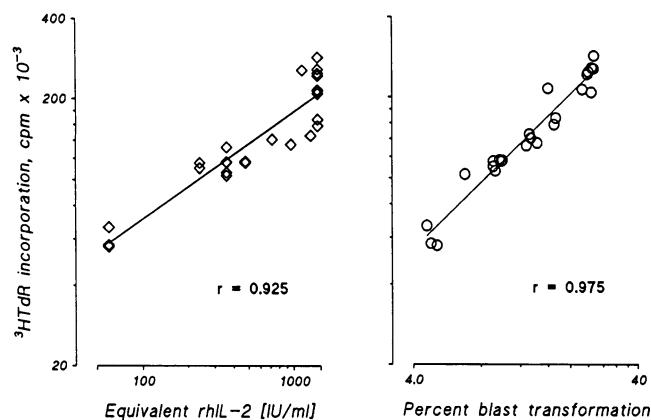
employing B-LCL (36), anti-CD3 mAb together with immobilized B7Ig fusion protein (8), or B7-transfected CHO cells (9) as stimuli for T cell activation. CD28 is a 44-kD homodimer expressed by CD4<sup>+</sup> and cytolytic CD8<sup>+</sup> T cells (41) that appears to activate human T cells by a direct effect on IL-2 production (16–19). Stimulation via CD28 induces a nuclear protein that binds to a specific site in the transcription enhancer region of the IL-2 gene (19). T cell stimulation via CD28 also prolongs the longevity of IL-2 mRNA (17, 18).

Another accessory ligand, ICAM-1 (CD54), is one of three molecules that binds the LFA-1 (CD11a:CD18) complex on T cells (6, 42). A second LFA-1 ligand, ICAM-2, does not yet have an established role in APC function (6), and the third ligand has not been characterized (42). Antigen-presentation is

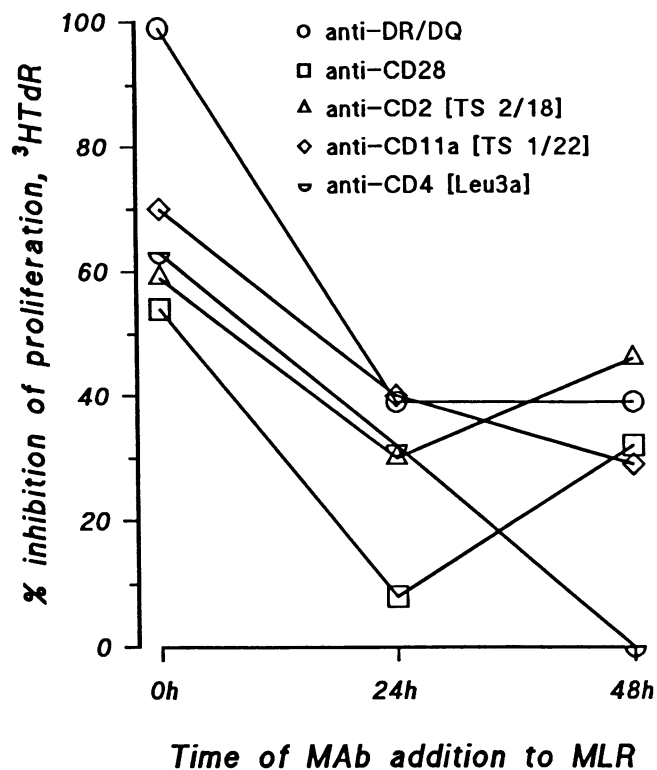
significantly more efficient by virtue of a functional ICAM-1:LFA-1 (CD54:CD11a) interaction (43, 44), and stimulation via either CD2 or CD3 increases LFA-1 (CD11a) binding avidity for ICAM-1 (CD54) (45, 46). ICAM-1 (CD54) and the  $\beta_2$  leukocyte integrins are widely distributed and are present on dendritic cells (CD54, CD11a/18, CD11c/18 [20]), T lymphocytes (CD11a/18 [6]), and T lymphoblasts (CD54 [6]; see also Results). CD4 is a co-recognition element for class II MHC (14, 47), engaging a specific MHC-peptide component together with the CD3/TCR complex. CD4 is coupled to a lymphocyte-specific tyrosine kinase (p56<sup>lck</sup>) that phosphorylates CD3 (48, 49) and may signal T lymphocytes in this manner. Lastly, CD2 is a pan-T cell surface antigen that binds the broadly distributed LFA-3 (CD58) ligand and is involved in both T cell adhe-

sion and activation (4, 5). CD2 signals T cell activation in conjunction with the CD3/TCR complex (50), via its cytoplasmic domain (5).

As different candidate, APCs (e.g., dendritic cells, monocytes, and B cells) are not equally capable of initiating primary T cell responses (20, 27), we speculate that their accessory ligand expression influences function in this respect. Dendritic cells express higher levels of B7/BB1, ICAM-1 (CD54), LFA-3 (CD58), and class I and II MHC products than other circulating leukocytes, suggesting an already activated phenotype in peripheral blood. We cannot exclude some degree of in vitro activation necessitated by the manipulations and culture involved in the purification of dendritic cells. However, previous experiments have demonstrated that a dendritic cell-containing fraction does not increase in stimulatory capacity during 1 or 2 d culture (27). We have also compared the different leukocyte subsets obtained during purification of dendritic cells, each handled identically, for their expression of B7/BB1. None of these populations (bulk PBMC, bulk  $Er^+$ , bulk  $Er^-$ , adherent  $Er^-$   $M\phi$  depleted of dendritic cells, or small B cells) expressed levels of B7/BB1 comparable to those of dendritic cells or control B-LCL. Only a trace subpopulation of adherent  $Er^-$   $M\phi$  expressed B7/BB1 very slightly above the isotype-matched control (data not shown). Unlike tonsillar B cells that express B7/BB1 after cross-linking of surface class II MHC products



**Figure 3.** Accessory ligand:receptor couple involvement in  $CD4^+$  lymphoblast transformation and IL-2 secretion after stimulation by allogeneic dendritic cells. Three sets of allogeneic MLRs ( $CD4^+$ :DC 25:1) were cultured in triplicate flat-bottomed microwells in the presence of mAbs to accessory ligands or receptors, singly and in combination, versus isotype-matched controls or no mAb. Supernatants were collected from one MLR set at 48 h and assayed for IL-2, based on their ability to support growth of a CTL2 line (33). Results are expressed in terms of IU rhIL-2/ml (1 Cetus Unit = 6 IU rhIL-2). The remaining two sets of MLRs were left in culture. One set was pulsed with  $1 \mu Ci^3H$ TdR/microwell for 8 h on d4  $\rightarrow$  5 to assess proliferation. The other set was simultaneously stained with 0.5% vol/vol propidium iodide and monitored cytofluorographically for viable blast transformation, based on increased forward scatter (compared with unstimulated  $CD4^+$  T cell controls) and negative FL2 fluorescence (nonviable cells are propidium iodide positive in the FL2 channel). The amount of  $^3H$ TdR incorporation was compared with the amount of IL-2 secretion ( $r = 0.925$ ) or percent blast transformation ( $r = 0.975$ ) for each MLR condition tested (e.g., presence or absence of mAbs at variable doses, as used in Fig. 2 and Table I). Representative of two experiments.



**Figure 4.** Effectiveness of anti-accessory receptor mAbs added at three sequential time points from the initiation of the dendritic cell-stimulated allogeneic  $CD4^+$  MLR. Three sets of allogeneic MLRs ( $CD4^+$ :DC 30:1) were cultured in triplicate flat-bottomed microwells in the presence or absence of the indicated mAbs or isotype-matched/T cell-reactive controls (each at  $10 \mu g/ml$  final). MABs were added to one set of MLR cultures at time 0 by precoating  $1.5 \times 10^5$   $CD4^+$  T cells directly in the microwells at  $4^\circ C$  for 30–60 min before adding allogeneic dendritic cells. Allogeneic  $CD4^+$  T cells and dendritic cells were combined in the other two sets of MLRs, initially without any mAb. At either 24 or 48 h after initiation of the MLR, the same mAbs used at time 0 were added to one or the other of the two remaining sets of MLR cultures. Each plate was placed at  $4^\circ C$  for 60 min before returning to culture at  $37^\circ C$ . All three sets of MLRs were pulsed with  $1 \mu Ci^3H$ TdR/microwell for 12 h d4  $\rightarrow$  5. Results are expressed in terms of percent inhibition of  $^3H$ TdR incorporation by each mAb, compared with respective isotype-matched controls, according to the time of mAb addition to the MLR. Representative of two experiments.

(36), the exact mechanism by which dendritic cells increase their expression of B7/BB1 and ICAM-1 (CD54) after binding antigen-specific T cells has not been defined. This phenotypic upregulation is specific for at least these two molecules, as LFA-3 (CD58), class II MHC, and CD45 expression remain relatively constant. Thus, while blood dendritic cells constitutively express an activated phenotype with regard to certain accessory ligands, they may upregulate other costimulatory molecules as a consequence of antigen-specific engagement between the CD3/TCR complex and peptide/MHC products. Our data do not permit specification of the order in which this occurs, nor does it exclude a role for any other accessory ligand:receptor pair involved in primary T cell activation.

To assess the biologic functions of these accessory ligands, we added mAbs with specificity for their receptors on the responding T cells. The absence of detectable FcR on blood den-



dritic cells permitted the use of intact immunoglobulin for this purpose, without risking mAb activation of the T cells (27). Blocking single accessory ligand:receptor couples only partially inhibited T cell responses in a primary allogeneic MLR. Complete inhibition required interference with at least three accessory ligand:receptor interactions. Alternatively, we observed a trend toward more complete inhibition by pairwise combinations of mAbs when the amount of antigen was reduced (i.e., fewer stimulatory allogeneic dendritic cells in the MLR). T cell proliferation, as well as IL-2 secretion and blast transformation, were all affected. Accessory signals seemed to be required in the first 24 h of a primary T cell response to alloantigen, since mAbs were substantially less inhibitory once proliferation had commenced. This was true whether we added anti-accessory receptor mAbs to MLR cultures at 24 or 48 h, compared with time 0, or whether these mAbs were added to antigen-specific T cell/dendritic cell clusters that had been separated from nonclustered T cells after 40 h initial culture (latter not shown). Anti-accessory molecule mAbs were less effective when added after the initiation of the MLR, either because costimulatory signals had already been delivered to the T cells, increased accessory ligand expression escaped inhibition, steric accessibility into the dendritic cell T lymphocyte clusters was limited, or the number of stimulatory dendritic cells was so high in the clusters as to restrict the inhibition that could be achieved. T cell-reactive, isotype-matched control mAbs against two other molecules involved in T cell signaling and activation, CD5 and CD45 (37), were never inhibitory under any condition. A similar analysis has been conducted of human tonsillar dendritic cells in T cell oxidative mitogenesis (51), and most of these same accessory couples contributed to function (except B7/BB1:CD28 which was unknown at that time). Those involved in cell-cell adhesion also blocked clustering, which we did not observe in the human dendritic cell-stimulated allogeneic MLR and which has also not been documented in the murine MLR (52).

CD3/TCR occupancy by antigen/MHC is insufficient to initiate primary T cell responses, as several additional costimulatory signals must be delivered by active APCs (1-3). This is underscored by our finding that complete inhibition of IL-2 secretion, blast transformation, and proliferation by primary T cells responding to alloantigen requires mAb block at several key points. These molecular couples are probably most integral to APC:T cell interactions when T cells are in a quiescent or unprimed state or when the amount of presented antigen is low, resulting in more stringent APC requirements (27, 32, 35, 53, 54). The use of mitogens or chronically stimulated T cell lines as responder populations may obscure relevant physiologic signals. B7/BB1, ICAM-1 (CD54), and LFA-3 (CD58) probably work in conjunction with antigen/MHC on dendritic cells, each delivering a distinct costimulatory signal to the T lymphocyte (3). B7/BB1 and ICAM-1 (CD54), although present on circulating dendritic cells, are upregulated after binding to T cells, presumably increasing T cell transcription and release of IL-2 via CD28 (8, 9, 16-19), as well as binding avidity via CD11a (45, 46). Adhesion should be bidirectional, as ICAM-1 (CD54) also appears on T lymphoblasts and can interact with LFA-1 (CD11a:CD18) on dendritic cells. B lymphoblastoid transformation (7, 26, 36, 38, 39) or monocyte exposure to  $\gamma$ -IFN (40) upregulates each cell's expression of many of these same accessory ligands. This should foster productive in-

teraction with T cells already sensitized by dendritic cells, maintaining and amplifying an effective immune response. Different APCs therefore do not differ qualitatively in their capacity to express these accessory ligands, but in their quantitative expression and the factors regulating same. We conclude that dendritic cells are unusual among circulating leukocytes in their comparably higher expression of accessory ligands, and that these are pertinent to the efficiency with which dendritic cells in small numbers elicit strong primary T cell proliferative and effector responses.

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