Published in final edited form as:

Transplantation. 1995 December 27; 60(12): 1539–1545.

BONE MARROW–DERIVED DENDRITIC CELL PROGENITORS (NLDC 145⁺, MHC CLASS II⁺, B7–1^{dim}, B7–2[–]) INDUCE ALLOANTIGEN-SPECIFIC HYPORESPONSIVENESS IN MURINE T LYMPHOCYTES^{1,2}

Lina Lu³, Delbert McCaslin³, Thomas E. Starzl³, and Angus W. Thomson^{3,4,5}

³ Pittsburgh Transplantation Institute and Department of Surgery, University of Pittsburgh.

⁴ Department of Molecular Genetics and Biochemistry, University of Pittsburgh.

Abstract

The functional maturation of dendritic cells (DC) and other antigen-presenting cells is believed to reflect the upregulation of cell surface major histocompatibility complex (MHC) class II and other T cell co-stimulatory molecules, especially the CD28 ligands B7–1 (CD80) and B7–2 (CD86). In this study, we propagated cells exhibiting characteristics of DC precursors from the bone marrow (BM) of BIO mice (H-2^b; I-A¹) in response to granulocyte-macrophage colony stimulating factor (GM-CSF). The methods used were similar to those employed previously to propagate DC progenitors from normal mouse liver. Cells expressing DC lineage markers (NLDC 145⁺, 33D1⁺ N418⁺) harvested from 8–10-day GM-CSF stimulated BM cell cultures were CD45⁺, heat-stable antigen⁺, CD54⁺, CD44⁺, MHC class II⁺, B7–1^{dim} but B7–2[–] (costimulatory molecule-deficient). Supplementation of cultures with interleukin-4 (IL-4) in addition to GM-CSF however, resulted in marked upregulation of MHC class II and B7–2 expression. These latter cells exhibited potent allostimulatory activity in primary mixed leukocyte cultures. In contrast, the cells stimulated with GM-CSF alone were relatively weak stimulators and induced alloantigen-specific hyporesponsiveness in allogeneic T cells (C3H; H-2^k; I-E⁺) detected upon re-stimulation in secondary MLR. This was associated with blockade of IL-2 production. Reactivity to third-party stimulators was intact. The hyporesponsiveness induced by the GM-CSF stimulated, costimulatory molecule-deficient cells was prevented by incorporation of anti-CD28 monoclonal antibody in the primary MLR and was reversed by addition of IL-2 to restimulated T cells. The findings show that MHC class II⁺ B7–2[–] cells with a DC precursor phenotype can induce alloantigen-specific hyporesponsiveness in vitro. Under the appropriate conditions, such costimulatory molecule-deficient cells could contribute to the induction of donor-specific unresponsiveness in vivo.

The functional maturation of dendritic cells (DC)^{*} and other antigen-presenting cells is now thought to reflect the upregulated expression of cell surface major histocompatibility

¹Presented at the 14th Annual Meeting of the American Society of Transplant Physicians, May 15–17, 1995, Chicago, IL.

²Supported in part by National Institutes of Health Grant DK 29961–14 and the Competitive Research Fund of the University of Pittsburgh Medical Center.

© 1995 by Williams & Wilkins

⁵ Address correspondence to Angus W. Thomson, D.Sc., University of Pittsburgh Medical Center, Pittsburgh Transplantation Institute, W1544 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15213..

^{*} Abbreviations: APC, antigen-presenting cell; BM, bone marrow; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction.

complex (MHC) class II and T cell costimulatory molecules, especially the CD28 ligands B7-1 (CD80) and B7-2 (CD86) (1,2). “Mature” DC are powerful inducers of naive T cell activation (3,4). It has been suggested, however, that their functionally “immature” precursors, that are deficient as inducers of allogeneic T cell responses, may have the capacity to function as tolerogenic cells (5-7).

The liver is comparatively rich in DC (8,9) and other leukocyte lineages compared with the heart or kidney. It is thought, however, to be the least immunogenic of whole organ allografts (10-12). In mice, it can be transplanted successfully between many strains despite MHC disparities and without immunosuppression. Moreover, it induces permanent, donor-specific unresponsiveness (12). This has led to the hypothesis that the liver possesses tolerogenic (as well as immunogenic) properties. Previously, we have reported that DC progenitors cultured from normal liver are capable of extensive proliferation in vitro in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) (7). These cells fail to stimulate allogeneic T cells in vitro, but migrate in vivo to T-dependent areas of allogeneic secondary lymphoid tissue (13). Therein at least some of the liver-derived cells strongly express donor MHC class II and persist for months, recapitulating the fate of donor-derived leukocytes up to 1 year after transplantation in non-immunosuppressed liver allograft recipients (12,14).

Recently, we have shown that small numbers of DC progenitors expressing donor (male) phenotype (MHC class I, II and Y-chromosome) can be propagated from the BM of unmodified (female) murine liver allograft recipients in response to GM-CSF (15). These findings have provoked questions about the function of donor-derived, DC lineage cells in relation to tolerance induction. Using techniques developed by Inaba et al. (16) and modified in this laboratory, we have propagated cells with characteristics of DC progenitors from mouse BM in response to GM-CSF. We report here on the function of these cells. They were found to be immunologically “immature” compared with BM-derived DC propagated in GM-CSF + IL-4. Whereas the GM-CSF + IL-4 induced DC were MHC class II⁺ B7-1⁺ B7-2⁺ and potent allostimulators, the cells stimulated with GM-CSF alone were MHC class II⁺ B7-1^{dim} B7-2⁻. Moreover, they induced alloantigen-specific hyporesponsiveness of purified T cells in primary mixed leukocyte reactions (MLR). These findings may be of importance in furthering our understanding of the mechanisms of tolerance induction following organ transplantation and in the development of new therapeutic strategies aimed at the prevention of their rejection.

MATERIALS AND METHODS

Animals

C57BL/10J (B10; H-2^b, I-A^b, I-E⁻), C3H/HeJ (C3H; H-2^k, I-A^k, I-E^k) and BALB/c (H-2^d, I-A^d, I-E⁺) mice 10–12 weeks old were obtained from The Jackson Laboratory, Bar Harbor, ME. They were maintained in a specific pathogen-free animal facility of the University of Pittsburgh Medical Center.

Propagation of bone marrow (BM)-derived cells

Bone marrow (BM) cell suspensions were prepared in RPMI-1640 (Gibco, Grand Island, NY) using conventional procedures. Cells (2×10^6) were cultured in 24-well plates in RPMI-1640 supplemented with 10% v/v fetal bovine serum (Gibco) and 500U (2 ng/ml recombinants) mouse GM-CSF (Schering-Plough, Kenilworth, NJ). In some experiments, culture medium was supplemented with r mouse IL-4 (Schering-Plough; 1000 U/ml) in addition to GM-CSF. The procedure used was modified (7) after that described by Inaba et al. (16) for the propagation of large numbers of DC progenitors from normal mouse BM.

The medium containing GM-CSF was refreshed every two days; after gentle swirling of the plates, half the “old” medium was aspirated and an equivalent volume of RPMI-1640 with GM-CSF $\pm$ IL-4 was added. An objective of this manipulation was to deplete nonadherent granulocytes without dislodging clusters of developing DC that attached loosely to a monolayer of firmly plastic-adherent macrophages. Morphologic and phenotypic analyses of typical single, nonadherent mononuclear cells released spontaneously from the clusters were performed between 6 and 10 days. These cells were tested for their capacity to stimulate allogeneic T cells.

Staining of cell surface and intracellular antigens

Cell surface staining was analyzed by cytofluorography using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Staining with primary hamster or rat monoclonal antibodies (mAbs), including rat anti-B7-1 and B7-2 (PharMingen, San Diego, CA) was followed by FITC-conjugated goat anti-hamster or mouse anti-rat Igs, as described (7,17). MHC class I and class II-positive cells were identified using biotin-conjugated mouse anti-mouse mAbs (PharMingen, San Diego, CA) with FITC streptavidin (Jackson Immunoresearch, West Grove, PA) as the secondary reagent. After staining, the cells were fixed in 1% paraformaldehyde in saline before analysis. MAbs to MHC class II antigens and to the 2A1 murine DC-restricted granule antigen were tested on cytospin preparations as described elsewhere (16,17). The specimens were fixed in acetone and stained with biotinylated mouse anti-MHC class II or rat anti-2A1 mAbs, followed by streptavidin biotin peroxidase complex (ABC-P; Boehringer Mannheim, Indianapolis, IN) or peroxidase-conjugated anti-rat Ig, respectively. The nuclei were counterstained with hematoxylin.

Mixed leukocyte reactions (MLR)

Splenocytes for use as responder cells were depleted of red blood cells by lysis in Tris-buffered NH_4Cl then T-cell enriched (>95%) by sequential removal of plastic-adherent cells (1 hr at 37°C) and high-affinity negative selection by passage (10 min at room temperature) through a mouse T cell enrichment column (R & D Systems, Minneapolis, MN). For MLR, 2×10^5 T cells were cultured with graded concentrations of γ -irradiated (20 Gy) allogeneic stimulator cells in 0.2 ml of medium for 3 days in 96-well, round-bottomed tissue culture plates in 5% CO_2 in air.

Testing for T cell hyporesponsiveness

For primary MLR, 2×10^6 T cells were incubated with 1×10^6 stimulators in 24-well plates in 2 ml of medium. In some experiments, hamster anti-CD28 mAb (PharMingen; 25 $\mu\text{g}/\text{ml}$) or control hamster IgG was added to the primary MLR. After 2 days, the cells were harvested, washed in medium, and depleted of Ia^+ cells with anti-I-A^b mAb (PharMingen) plus lowtoxicity rabbit complement (Accurate Chemical & Scientific, West-bury, NY) (7). Viable cells were isolated by centrifugation over Ficoll-Isopaque for 15 min at 350 $\times g$ and rested in medium for 2 days in the absence of stimulators. The pretreated T cells were then restimulated with freshly isolated γ -irradiated spleen cells in the absence or presence of r human IL-2 (Gibco; 50U/ml) in a secondary, 3-day MLR in 96-well round-bottomed plates. For the final 18 hr, 10 μl [^3H]TdR (1 μCi) was added to each well. Cultures were harvested onto glass fiber disks using a multiple cell harvester and the degree of thymidine incorporation was determined in a liquid scintillation counter. Results were expressed as mean cpm $\pm$ 1 SD.

IL-2 assay

IL-2 levels in MLR culture supernatants were determined by the CTLL assay. CTLL cells (ATCC, Rockville, MD; $5 \times 10^3/\text{well}$) in exponential growth phase were suspended in

RPMI-1640 containing 10% FCS and incubated in flat-bottomed microtiter wells for 48 hr with dilutions of these supernatants or various concentrations of standard rIL-2 (Gibco). [³H]TdR incorporation was assessed as described above. IL-2 production was determined by comparing the CTLL [³H]TdR incorporation supported by supernatants with that supported by known concentrations of rIL-2. Results were expressed as mean U IL-2/ml $\pm$ 1 SD.

RESULTS

Propagation of cells from bone marrow

In previous studies, aggregates of growing DC progenitors have been described in GM-CSF-stimulated cultures established from mouse BM (16), blood (18), spleen (17), or liver (7). Similar techniques were used to propagate DC progenitors from the BM of B10 mice. In view of reports that GM-CSF + IL-4 is much more effective than GM-CSF alone in inducing DC maturation (19), this cytokine combination was also tested. Whether GM-CSF was used alone or in combination with IL-4, the floating cells released from the clusters and harvested at 6–10 days exhibited morphological characteristics of DC; dendritic morphology was especially prevalent in cells stimulated with both cytokines.

Immunophenotypic analysis of GM-CSF stimulated cells

Cytospins were used to further characterize the released cells. By day 10, the majority expressed moderate levels of MHC class II. Granulocytes were rare. Most cells also strongly expressed the DC-restricted cytoplasmic granule antigen 2A1 (16,17). To further characterize the surface immunophenotype of the cells released from proliferating aggregates, flow cytometric analysis was performed after 8–10 days of culture in GM-CSF. Staining for cells of lymphoid lineage, including NK cells was absent. As shown in Figure 1, however, the floating cells in 8–10-day BM-derived cultures expressed surface antigens that are known to be associated with mouse DC. These included CD45 (leukocyte-common antigen), MHC class I, heat stable antigen (J11D), CD54 (intercellular adhesion molecule-1; ICAM-1), GD11b (MAC-1), and CD44 (nonpolymeric determinant of Pgp.1 glycoprotein). In addition, staining of moderate intensity was observed for the mouse DC-restricted cell surface markers NLDC-145 (interdigitating cells), 33D1 and CD11c (N418; β 2-integrin), for the macrophage antigen F4/80, and for Fc γ RII (CD32), as described previously (16) for BM-derived DC. Expression of the low-affinity IL-2 receptor (CD25) could not be demonstrated. In agreement with the cytospin results, the GM-CSF-stimulated BM-derived cells expressed moderate levels of cell surface MHC class II (I-A^b).

Enhanced expression of MHC class II and of B7-1 and B7-2 by GM-CSF + IL-4-stimulated BM-derived cells

Cells stimulated with GM-CSF either alone or in combination with IL-4 were harvested after 8–10 days and compared for the expression of surface markers linked with allostimulatory activity. A marked difference was consistently observed between the two cell populations with respect to the intensity of expression of MHC class II and the CD28 ligands, B7-1 (CD80) and B7-2 (CD86). As shown in Figure 2, MHC class II was positive and B7-1 expression was low, whereas B7-2 was negligible/dim on cells stimulated with GM-CSF (500U/ml) alone. An increase in GM-CSF concentration from 500–1000 U/ml augmented the intensity of staining for each of these molecules (data not shown). However, expression of MHC class II, and especially B7-2, was markedly and consistently upregulated on cells treated with both cytokines (Fig. 2). Similar results were obtained with GM-CSF + IL-4-stimulated BM-derived cells from B10BR and C3H mice. The GM-CSF-stimulated MHC class II⁺ B7-1^{dim} B7-2⁻ and the GM-CSF + IL-4-induced MHC class II⁺ B7-1⁺ B7-2⁺ populations are subsequently referred to herein as B7-2⁻ and B7-2⁺, respectively.

MLR stimulatory activity of B7-2⁻ and B7-2⁺ cells

As shown in Figure 3, the GM-CSF + IL-4-stimulated B10 BM-derived cells that were MHC class II⁺, B7-1⁺ and B7-2⁺ were highly potent inducers of primary allogeneic (C3H) T cell responses in 3-day primary mixed leukocyte cultures. The possibility that this high MLR stimulatory activity was due to “carry-over” of IL-4 was excluded as GM-CSF + IL-4-stimulated syngeneic B7-2⁺ cells did not induce T cell proliferation. On the basis both of immunophenotype and T cell stimulatory activity, the B7-2⁺ cells were considered to be predominantly “mature” DC. In contrast, GM-CSF stimulated MHC class II⁺ B7-2⁺ BM-derived cells were much poorer inducers of allogeneic T cell activation and exhibited a comparatively low level of MLR-stimulatory activity. This was consistent with their low level of cell surface B7-2 and similar to that of freshly isolated bulk B10 spleen cells. The B7-2⁻ cells were therefore considered to be functionally “immature,” DC progenitors.

Induction of alloantigen-specific hyporesponsiveness by B7-2⁻ BM-derived cells

We considered that deficiency in cell surface expression of the B7-2 molecule might be associated with the induction of hyporesponsiveness/anergy by these cells. We therefore tested the capacity of the B7-2⁻ cells to induce alloantigen-specific anergy during a primary MLR. Purified C3H splenic T cells were stimulated initially with either freshly isolated B10 bulk spleen cells or GM-CSF stimulated (B7-2⁻) or GM-CSF + IL-4-stimulated (B7-2⁺) B10 BM cells; after resting for 2 days, the primed C3H T cells were restimulated in a secondary MLR with fresh B10, C3H (syngeneic) or BALB/c (third-party) splenocytes. As shown in Figure 4, the T lymphocytes stimulated with B7-2⁺ B10 cells in a primary MLR displayed strong proliferative responses when restimulated with B10 spleen cells. In contrast, T cells exposed initially to B7-2⁻ B10 BM cells responded only weakly upon restimulation with B10 splenocytes and at a lower level than C3H T cells primed with fresh B10 splenocytes. The same T cells however, displayed proliferative responses to third-party (BALB/c) spleen cells (Fig. 5), indicating that the hyporesponsiveness was donor-specific.

Anti-CD28 mAb prevents hyporesponsiveness induced by B7-2⁻ BM-derived cells

It is recognized that blockade of the CD28 costimulatory pathway can induce alloantigen-specific hyporesponsiveness (20) or tolerance (21). We therefore determined whether the addition of anti-CD28 mAb to primary MLR cultures could reverse the capacity of B7-2⁻ cells to induce T cell hyporesponsiveness. The presence of anti-CD28 mAb significantly increased the allostimulatory function of the costimulatory molecule-deficient cells. Furthermore, as shown in Figure 6, priming of allogeneic T cells with B7-2⁻ cells in the presence of anti-CD28 prevented hyporesponsiveness in secondary MLR.

Hyporesponsiveness induced by B7-2⁻ cells is associated with impaired IL-2 production

To determine whether the induction of hyporesponsiveness in C3H T cells was linked to blockade of IL-2 production, levels of IL-2 were determined in supernatants harvested from B10 restimulated T cells primed initially with B10 splenocytes, B7-2⁻ B10 cells, or B7-2⁺ B10 cells in the presence of anti-CD28. As shown in Table 1, C3H T cells primed with B7-2⁻ cells failed to produce IL-2 in 2° MLR. The presence of anti-CD28 in the 1° MLR however, prevented subsequent unresponsiveness.

T cells primed by B7-2⁻ cells respond normally to alloantigen + IL-2 in 2° MLR

There is evidence that IL-2 can prevent or reverse hyporesponsiveness in T cells anergized by antigen stimulation in the absence of costimulatory signals (20). Table 2 demonstrates that the hyporesponsive T cells primed by B7-2⁻ BM-derived cells responded normally to alloantigen + exogenous IL-2 (50 U/ml) added at the outset of secondary stimulation. Indeed, T cell proliferation was enhanced. Collectively, these data strongly suggest the

importance of an IL-2 production defect in the induction of the hyporesponsive state. They also indicate that IL-2R expression was not affected, as the T cells responded vigorously to IL-2 in secondary MLR.

DISCUSSION

In contrast to classic mature DC (3), NLDC 145⁺, MHC class II⁺, B7-1^{dim}, B7-2⁻ mouse BM-derived cells propagated in GM-CSF induced alloantigen-specific hyporesponsiveness in T cells upon rechallenge in vitro. This hyporesponsive state was inhibited by stimulation via the CD28 pathway and reversed by exogenous IL-2. It was also found in this study that BM cells stimulated with GM-CSF + IL-4 exhibited an overall similar phenotype to those propagated in GM-CSF alone, but stained intensely for B7-2 in addition to enhanced B7-1 and high MHC class II expression. In contrast to the B7-2⁻ population, these cells were very potent allostimulators. The findings are consistent with those of Hathcock et al. (22), who showed that quantitative differences in B7-1 and B7-2 expression on murine APC (B cells) could profoundly affect their contribution to costimulatory function (22).

The influence of cytokines and cytokine combinations on B7-2 expression and DC maturation has been reported. Thus Larsen et al. (1) found that GM-CSF or IFN- γ induced increased expression of B7-2 on cultured murine spleen DC and that GM-CSF also increased the expression of ICAM-1 and HSA. Only partial reduction of B7-2 expression was achieved, however, by the addition of anti-GM-CSF to skin cultures (2). Recently, Sallusto and Lanzavecchia (19) have shown that cultured human blood DC expanded in GM-CSF + IL-4 are highly stimulatory in MLR and considerably more potent than those induced with GM-CSF alone. The present finding, that GM-CSF plus IL-4 markedly upregulated cell surface B7-2 expression and allostimulatory activity suggests that IL-4 may also be an important costimulatory cytokine for the functional maturation of BM DC. Interestingly, although mouse small resting B cells induce anergy following exposure to IL-4, they express B7-1 and B7-2 molecules and stimulate lymphokine production and proliferation in Th1 clones (23). Blocking of B7-1, however, does not affect the ability of IL-4-treated B cells to stimulate Th1 cell activation (22).

MHC class II⁺ APC that are costimulatory molecule deficient may (as is the case with inhibition of B7-2 [22]) allow TCR-mediated signaling events to occur but inhibit the distinct costimulatory signal(s) necessary for optimal cytokine production and cytokine-dependent T cell proliferation. Thus, antigen presentation by APC that are unable to deliver adequate costimulatory signals induces neither of the transcription factors NF-AT or NF- κ B (24). Absence of IL-2 expression during the induction of T cell anergy may be attributed to the normal induction (during the stimulation response) of a repressor factor(s) and the establishment of a stable, nontranscriptionally active IL-2 gene complex. As shown by others (25) and in the present study, the induction of hyporesponsiveness or anergy in murine T cells can be prevented by CD28 stimulation using mAb. Several adhesion receptor-ligand pairs however, such as ICAM-1-LFA-1 can costimulate T cell proliferation. Nevertheless, B7 but not ICAM-1 induces detectable IL-2 secretion and prevents the induction of alloantigen-specific anergy (26). Expression of both ICAM-1 and HSA on the B7-2⁻ cells in our system may account for T cell hyporesponsiveness as opposed to the induction of anergy.

The induction of irreversible T cell anergy to donor-specific alloantigens in vivo (tolerance) would, in theory, eliminate the need for potentially toxic, nonspecific immunosuppressive agents that currently constitute first choice antirejection therapy. There has thus been considerable interest in evidence that blockade of the B7-CD28 T cell costimulatory pathway is both necessary and sufficient to induce antigen-specific T cell anergy (27).

Although the costimulatory signals delivered to T cells by APCs are neither antigen-specific nor MHC-restricted, they determine the outcome of TCR signaling as they mediate cytokine secretion. Co-ligation of the T cell CD28/CTLA-4 receptors (an essential stimulus of T cell activation) (28) by the APC counter-receptors B7/BB1 (CD80) or the recently identified B70/B7-2 (CD86) (29-31) results in IL-2 production and T cell proliferation (28,29,31). It is thought that this is achieved by increased IL-2 mRNA transcription (32,33) or by increased mRNA stability (34). Elicitation of strong IL-2 production by T cells has long been recognized as a characteristic of antigen-presenting DC (35,36).

While functional “immaturity” is evident in freshly isolated DC from mouse BM, spleen (37), and skin (38), which express little or no B7-2, and from human blood (39), functional maturation develops in vitro in response to appropriate cytokines. This is correlated with marked upregulation of B7 molecules (40), in particular B7-2 (2). Although DC express several adhesion and costimulatory molecules (CD11a, CD44, CD54, CD48/58, CD80 and CD86), B7-2 appears to be the major CD28/CTLA-4 ligand and accounts for the costimulation provided by this pathway during in vitro immune responses. Indeed, in a recent study, anti-CD80 (B7-1) mAbs failed to inhibit human dendritic Langerhans cell-induced alloactivation of T cells (41). Early and sustained expression of B7-2 mRNA (within 8 hr) (before maximal B7-1 expression) in activated murine B cells (42), suggests that it may provide a critical signal (within the first 24 hr) involved in the decision between activation or inactivation of T cells within 24 hr of stimulation (43,44). Moreover, APCs from mice in which the B7-1 gene has been “knocked out” express B7-2 and are competent APCs (42).

T cell costimulation mediated by CD28 is important in the mediation of transplant rejection and the development of new therapeutic immunosuppressive strategies. Thus combination of anti-B7 mAb with cyclosporine A induces alloantigen-specific anergy in vitro (45). CTLA4-Ig, a fusion protein that blocks the CD28-mediated costimulatory signal inhibits T cell proliferation in allogeneic MLR (20,40), and induces hyporesponsiveness to alloantigen in vitro (20). Moreover, it prolongs MHC-mismatched cardiac allograft survival in rats (21,46) and induces donor-specific transplantation tolerance to skin grafts in cardiac-allografted mice (47). Propagation of B7-2⁺ cells with characteristics of DC progenitors and the capacity to induce alloantigen-specific hyporesponsiveness has significant in vivo implications. We have shown that DC progenitors can be propagated from the liver in response to GM-CSF. Conceivably, the reduced immunogenicity of liver allografts may be linked, at least in part, to the release of potentially T cell anergizing APC from the liver, a pseudohe-mopoietic organ (48). This is likely to occur under the influence of locally-produced GM-CSF (49) and possibly other growth promoting cytokines. Failure of the B7-2⁺ cells to induce complete unresponsiveness (anergy) in vitro, however, suggests that considerations of the cellular therapy of allograft rejection using donor-derived DC progenitors should include the use of adjunctive immunosuppression, implementing drugs or molecular approaches geared to inhibit costimulatory molecule expression on APC.

Acknowledgments

We thank Ms. Frances Chambers for skilled technical assistance, Dr. Walter Storkus and Dr. Susan McCarthy for review of the manuscript, Dr. Satwant Narula (Schering-Plough, Kenilworth, NJ) for providing cytokines, and Ms. Shelly Conklin for secretarial support.

REFERENCES

1. Larsen CP, Ritchie SC, Hendrix R, Linsley PS, Hathcock KS, Hodes RJ, Lowry RP, Pearson TC. Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. *J Immunol* 1994;152:5208. [PubMed: 7514631]

2. Inaba K, Witmer-Pack M, Inaba M, et al. The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. *J Exp Med* 1994;180:1849. [PubMed: 7525841]
3. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991;9:271. [PubMed: 1910679]
4. Austyn, JM. The initiation of immune responses and allograft rejection. In: Rose, ML.; Yacoub, MH., editors. *Immunology of heart and lung transplantation*. Little, Brown; Boston: 1993. p. 22
5. Starzl TE, Demetris AJ, Murase N, Ildstad S, Ricordi C, Trucco M. Cell migration, chimerism, and graft acceptance. *Lancet* 1992;339:1579. [PubMed: 1351558]
6. Steinman RM, Inaba K, Austyn JM. Donor-derived chimerism in recipients of organ transplants. *Hepatology* 1993;17:1153. [PubMed: 8514265]
7. Lu L, Woo J, Rao AS, et al. Propagation of dendritic cell progenitors from normal mouse liver using GM-CSF and their maturational development in the presence of type-1 collagen. *J Exp Med* 1994;179:1823. [PubMed: 8195710]
8. Hart DNJ, Fabre JW. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissue of rat heart and other tissues but not brain. *J Exp Med* 1981;153:347. [PubMed: 6943285]
9. Woo J, Lu L, Rao AS, Li Y, Subbotin V, Starzl TE, Thomson AW. Isolation, immunophenotype and allostimulatory activity of murine liver dendritic cells. *Transplantation* 1994;58:484. [PubMed: 8073517]
10. Starzl TE, Marchioro TL, Porter KA, et al. Factors determining short- and long-term survival after orthotopic liver homotransplantation in the dog. *Surgery* 1965;58:131. [PubMed: 14305148]
11. Calne RY, Sells RA, Pena JR, Davis DR, Millard PR, Herbertson BM, Binns RM, Davies DAL. Induction of immunological tolerance by porcine liver allografts. *Nature (Lond.)* 1969;233:472. [PubMed: 4894426]
12. Qian S, Demetris AJ, Murase N, Rao AS, Fung JJ, Starzl TE. Murine liver allograft transplantation: tolerance and donor cell chimerism. *Hepatology* 1994;19:916. [PubMed: 8138266]
13. Thomson AW, Lu L, Subbotin VM, Li Y, Qian S, Rao AS, Fung JJ, Starzl TE. In vitro propagation and homing of liver-derived dendritic cell progenitors to lymphoid tissues of allogeneic recipients. *Transplantation* 1995;59:544. [PubMed: 7878760]
14. Demetris AJ, Murase N, Fujisaki S, Fung JJ, Rao AS, Starzl TE. Hematolymphoid cell trafficking, chimerism and tolerance after liver, bone marrow, and heart transplantation: rejection, GVH disease and the merging of the immune systems. *Transplant Proc* 1993;25:3337. [PubMed: 7505503]
15. Lu L, Rudert WA, Qian S, et al. Growth of donor-derived dendritic cells from the bone marrow of murine liver allograft recipients in response to granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1995;182:379. [PubMed: 7629500]
16. Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cell from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176:1693. [PubMed: 1460426]
17. Lu L, Hsieh M, Oriss TB, et al. Generation of dendritic cells from mouse spleen cell cultures in response to GM-CSF: immunophenotypic and functional analyses. *Immunology* 1995;84:127. [PubMed: 7890296]
18. Inaba K, Steinman RM, Pack MW, et al. Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med* 1992;175:1157. [PubMed: 1569392]
19. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α . *J Exp Med* 1994;179:1109. [PubMed: 8145033]
20. Tan P, Anasetti C, Hansen JA, et al. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J Exp Med* 1993;177:165. [PubMed: 7678111]
21. Turka LA, Linsley PS, Lin H, et al. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc Natl Acad Sci USA* 1992;89:11102. [PubMed: 1332070]

22. Hathcock KS, Laszlo G, Pucillo C, Linsley P, Hodes RJ. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J Exp Med* 1994;180:631. [PubMed: 7519245]
23. Stack RM, Lenschow DJ, Gray GS, Bluestone JA, Fitch FW. IL-4 treatment of small splenic B cells induces costimulatory molecules B7-1 and B7-2. *J Immunol* 1994;152:5723. [PubMed: 7515912]
24. Go C, Miller J. Differential induction of transcription factors that regulate the interleukin 2 gene during anergy induction and restimulation. *J Exp Med* 1992;175:1327. [PubMed: 1569401]
25. Harding F, McArthur J, Gross J, Raulet D, Allison J. CD28-mediated signaling costimulates murine T cells and prevents induction of anergy in T cell clones. *Nature* 1992;356:607. [PubMed: 1313950]
26. Boussiotis VA, Freeman GJ, Gray G, Gribben J, Nadler LM. B7 but not ICAM-1 co-stimulation prevents the induction of human alloantigen specific tolerance. *J Exp Med* 1993;178:1753. [PubMed: 7901318]
27. Boussiotis VA, Gribben JG, Freeman GJ, Nadler LM. Blockade of the CD28 co-stimulatory pathway: a means to induce tolerance. *Curr Opin Immunol* 1994;6:797.
28. Linsley P, Ledbetter J. The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* 1993;11:191. [PubMed: 8386518]
29. Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL, Somoza C. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* 1993;366:76. [PubMed: 7694153]
30. Hathcock KS, Laszlo G, Pucillo C, Linsley P, Hodes RJ. Comparative analysis of B7-1 and B7-2 co-stimulatory ligands: expression and function. *J Exp Med* 180:631. [PubMed: 7519245]
31. Freeman GJ, Gribben JG, Boussiotis VA, et al. Cloning of B7-2: a CTLA4 counter-receptor that co-stimulates human T-cell proliferation. *Science* 1993;262:909. [PubMed: 7694363]
32. Fraser JD, Irving BA, Crabtree GR, Weiss A. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* 1991;251. [PubMed: 17794670]
33. Verweji CL, Geerts M, Aarden LA. Activation of interleukin-2 gene transcription via the T-cell surface molecule CD28 is mediated through an NF-KB-like response element. *J Biol Chem* 1991;266:14. 179.
34. Lindsten T, June CH, Ledbetter JA, Stella G, Thompson CB. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* 1989;244:339. [PubMed: 2540528]
35. Klinkert WEF, Labadie JH, O'Brien JP, Beyer CF, Bowers WE. Rat dendritic cells function as accessory cells and control the production of a soluble factor required for mitogen responses of T lymphocytes. *Proc Natl Acad Sci USA* 1978;77:5414. [PubMed: 6968911]
36. Austyn JM, Steinman RM, Weinstem DE, Granelli-Piperno A, Palladino MA. Dendritic cells initiate a two-stage mechanism for T lymphocyte proliferation. *J Exp Med* 1983;157:1101. [PubMed: 6300278]
37. Crowley MT, Inaba K, Witmer-Pack MD, Gezelter S, Steinman RM. Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J Immunol Methods* 1990;133:55. [PubMed: 2145370]
38. Schuler G, Steinman RM. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med* 1985;161:526. [PubMed: 3871837]
39. O'Doherty U, Steinman RM, Peng M, et al. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J Exp Med* 1993;178:1067. [PubMed: 8102389]
40. Larsen CP, Ritchie SC, Pearson TC, Linsley PS, Lowry RP. Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. *J Exp Med* 1992;176:1215. [PubMed: 1328465]
41. Caux C, Vanbervliet B, Massacrier C, et al. B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J Exp Med* 1994;180:1841. [PubMed: 7525840]
42. Freeman GJ, Borriello F, Hodes RJ, et al. Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. *Science* 1993;262:907. [PubMed: 7694362]

43. Gimmi CD, Freeman GJ, Gribben JG, Gray G, Nadler LM. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 co-stimulation. *Proc Natl Acad Sci USA* 1993;90:6586. [PubMed: 7688125]
44. Boussiotis VA, Freeman GJ, Gribben JG, Daley J, Gray G, Nadler LM. Activated human B lymphocytes express three CTLA-4 counter-receptors that co-stimulate T-cell activation. *Proc Natl Acad Sci USA* 1993;90:11. 059. [PubMed: 8093556]
45. Van Gool SW, de Boer M, Ceuppens JL. The combination of anti-B7 monoclonal antibody and cyclosporin A induces alloantigen-specific anergy during a primary mixed lymphocyte reaction. *J Exp Med* 1994;179:715. [PubMed: 7507511]
46. Lin H, Boiling SF, Linsley PS, Wei R-Q, Gordon D, Thompson CB, Turka LA. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J Exp Med* 1993;178:1801. [PubMed: 8228826]
47. Pearson TC, Alexander DZ, Winn KJ, Linsley PS, Lowry RP, Larsen CP. Transplantation tolerance induced by CTLA4-Ig. *Transplantation* 1994;57:1701. [PubMed: 8016872]
48. Sakamoto T, Saizawa T, Mabuchi A, Norose Y, Shoji T, Yokomuro K. The liver as a potential hematolymphoid organ examined from modifications occurring in the systemic and intrahepatic hematolymphoid system during liver regeneration after partial hepatectomy. *Reg Immunol* 1992;4:1. [PubMed: 1571227]
49. Sakamoto T, Mabuchi A, Kuriya S-I, et al. Production of granulocyte-macrophage colony-stimulating factor by adult murine parenchymal liver cells (hepatocytes). *Reg Immunol* 1990/1991;3:260. [PubMed: 2129610]

Figure 1.

FACSscan immunophenotypic profiles of GM-CSF-stimulated B10 (H-2^b) mouse BM-derived cells released from cell aggregates in liquid culture (day 10) and examined using rat, hamster, or mouse mAbs. Further details are provided in *Materials and Methods* or have been described elsewhere (7). Unshaded profiles denote appropriate Ig isotype subclass controls. Note the absence of expression of lymphoid cell markers and the staining for three murine DC-restricted antigens NLDC 145, N418 (CD11c) and 33D1. The result is representative of 4 separate experiments.

Figure 2.

Expression of MHC class II (I-A^b), B7-1 and B7-2 on (above) 8-day GM-CSF and (below) 8-day GM-CSF + IL-4-stimulated B10 BM-derived bone marrow cells. The result is representative of 3 separate experiments.

Figure 3.

The MLR stimulatory capacity of B10 (H-2^b) mouse BM-derived cells (day 8) propagated in response to (○) GM-CSF (B7-2⁻) or (●) GM-CSF + IL-4 (B7-2⁺). Graded concentrations of washed, γ -irradiated stimulator cells were cultured with 2×10^5 purified allogeneic (C3H; H-2^k) T cells. The stimulatory activity of freshly-isolated (Δ) B10 and ($\blacksquare$) C3H (syngeneic) spleen cells and of ($\square$) GM-CSF + IL-4 stimulated (C3H) syngeneic cells is also shown. Cells were pulsed with [³H]TdR for the final 18 hr of 3-day cultures. The results are expressed as mean counts per minute (cpm) $\pm$ 1 SD and are representative of three separate experiments.

Figure 4.

Stimulation with B7-2⁻ BM-derived cells during a primary MLR induces hyporesponsiveness upon restimulation in secondary MLR. C3H splenic T cells ($2 \times 10^6/\text{ml}$ at 1:2 [S:R]) ratio were stimulated during a 2-day primary MLR with γ -irradiated ($\circ$) GM-CSF-stimulated B10 BM-derived cells (B7-2⁻) ($\bullet$) GM-CSF + IL-4-stimulated B10 BM-derived cells (B7-2⁺), or (Δ) fresh B10 spleen cells. The T cells were repurified and rested for 2 days, then restimulated for 3 days with graded concentrations of freshly isolated B10 spleen cells. The results are expressed as mean cpm $\pm$ 1 SD and are representative of four separate experiments.

Figure 5.

T cells primed by B7-2⁻ BM-derived cells are specifically hyporesponsive to donor alloantigens. C3H splenic T cells were stimulated during a 2-day primary MLR with either (△) freshly-isolated B10 bulk spleen cells or (○) B7-2⁻ B10 cells. They were repurified, rested for 2 days, and restimulated for 3 days with C3H, B10 or BALB/c (third party) spleen cells. Results are expressed as mean counts per minute (cpm) ± 1 SD and are representative of 3 separate experiments.

Figure 6.

Addition of anti-CD28 mAb (25 μ g/ml) to primary MLR can prevent T cell hyporesponsiveness induced by B7-2⁻ BM-derived cells. C3H splenic T cells were stimulated during a 2-day primary MLR with (□), fresh B10 spleen cells; (■), B7-2⁻ B10 cells; (▨) B7-2⁻ B10 cells + hamster IgG or (▤), or B7-2⁻ B10 cells + anti-CD28. The T cells were repurified, rested for 2 days, and restimulated in 3-day secondary MLR with freshly isolated B10 spleen cells. The results are expressed as mean counts per minute (cpm) $\pm$ 1 SD and are representative of three separate experiments.

Table 1

Hyporesponsiveness induced by B7-2 B10 BM cells in primary MLR is associated with impaired IL-2 production and is prevented by anti-CD28

1° MLR:stimulators	2° MLR:B10 stimulators ($\times 10^3$)		
	25	50	100
C3H spleen cells (syngeneic)	0.67 $\pm$ 0.17 ^a	1.22 $\pm$ 0.26	2.49 $\pm$ 0.18
B10 spleen cells	1.38 $\pm$ 0.17	4.68 $\pm$ 0.53	5.72 $\pm$ 0.11
B7-2 ⁻ B10 BM cells	0.84 $\pm$ 0.13	1.30 $\pm$ 0.10	1.99 $\pm$ 0.20
B7-2 ⁻ B10 BM cells + hamster IgG	ND ^b	1.75 $\pm$ 0.14	1.82 $\pm$ 0.14
B7-2 ⁻ B10 BM cells + anti-CD28	2.15 $\pm$ 0.11	7.55 $\pm$ 1.10	7.76 $\pm$ 1.44

^aCTLL cell proliferation (U IL-2/ml).

^bND = not done.

Table 2
C3H T cells primed by B7-2⁻ B10 BM cells respond normally to alloantigen + IL-2 in 2° MLR: [³H] TdR uptake (cpm × 10³ ± 1 SD)

1° MLR:stimulators	IL-2 (50U/ml)	2° MLR:No. B10 stimulators ^a (×10 ³)			
		1 ^b	25	50	100
B10 spleen cells	–	7.86±0.68	18.54±0.44	23.81±0.46	25.78±0.59
	+	ND ^c	247.47±41.12	240.00±11.74	231.81±11.06
B7-2 ⁻ B10 BM cells	–	10.79±0.28	9.89±0.51	11.34±0.38	13.86±0.78
	+	ND	338.01±10.50	341.24±46.10	291.32±44.05

^a Fresh spleen cells.
^b 10⁵ C3H (syngeneic) spleen cell stimulators used as control.
^c ND = not done.