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COSTIMULATORY MOLECULE-DEFICIENT DENDRITIC CELL PROGENITORS (MHC CLASS II$^+$, CD80^{dim}, CD86$^-$) PROLONG CARDIAC ALLOGRAFT SURVIVAL IN NONIMMUNOSUPPRESSED RECIPIENTS[1,2]

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We have shown previously that granulocyte-macrophage colony-stimulating factor-stimulated mouse bone marrow-derived MHC class II$^+$ dendritic cell (DC) progenitors that are deficient in cell surface expression of the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) can induce alloantigen-specific T-cell anergy in vitro. To test the in vivo relevance of these findings, 2×10^6 B10 (H2^b) mouse bone marrow-derived DC progenitors (NLDC 145$^+$, MHC class II$^+$, B7-1^{dim}, B7-2$^{-/dim}$) that induced T-cell hyporesponsiveness in vitro were injected systemically into normal C3H (H2^k) recipients. Seven days later, the mice received heterotopic heart transplants from B10 donors. No immunosuppressive treatment was given. Median graft survival time was prolonged significantly from 9.5 to 22 days. Median graft survival time was also increased, although to a lesser extent (16.5 days), in mice that received third-party (BALB/c; H2^d) DC progenitors. Ex vivo analysis of host T-cell responses to donor and third-party alloantigens 7 days after the injection of DC progenitors (the time of heart transplant) revealed minimal anti-donor mixed leukocyte reaction and cytotoxic T lymphocyte reactivity. These responses were reduced substantially compared with those of spleen cells from animals pretreated with "mature" granulocyte-macrophage colony-stimulating factor + interleukin-4-stimulated DC (MHC class II^{bright}, B7-1$^+$, B7-2^{bright}), many of which rejected their heart grafts in an accelerated fashion. Among the injected donor MHC class II$^+$ DC progenitors that migrated to recipient secondary lymphoid tissue were cells that appeared to have up-regulated cell surface B7-1 and B7-2 molecule expression. This observation may explain, at least in part, the temporary or unstable nature of the hyporesponsiveness induced by the DC progenitors in nonimmunosuppressed recipients.

The discovery in 1992 of multilineage leukocyte microchimerism many years after organ transplantation (*1*, *2*) implied the migration and survival of donor progenitor or even stem cells within recipient tissues. A prominent donor leukocyte, both in these clinical observations and in experimental animal studies (*3*, *4*), has been the dendritic cell (DC*). These bone marrow (BM)-derived leukocytes are the most potent known antigen-presenting cells (APC). They have the unique capacity to activate resting, naive T lymphocytes (*5*). In addition, however, tolerogenic properties of DC have been described both in vitro (*6*, *7*) and in vivo (*8*, *9*). Recently, we reported that donor-derived DC progenitors can be propagated in vitro from the BM of unmodified mice that permanently accept MHC-incompatible liver transplants, but not from the lymphoid tissue of animals that acutely reject heart grafts (*10*). The in vivo functional relevance of donor-derived DC progenitors has not been established. It has been suggested that, in sufficient numbers, these chimeric APC may play a role in the induction of transplantation tolerance (*1-4*, *10*, *11*).

The functional maturation of DC and other APC, such as macrophages or activated B cells, has been shown to depend on the cytokine-induced up-regulation of cell surface MHC class II and T-cell "costimulatory" molecules, particularly the CD28 ligands B7-1 (CD80) and B7-2 (CD86) (*12*, *13*). We hypothesized that in accordance with the "two-signal" hypothesis of T-cell activation (*14*), failure of MHC class II$^+$ DC progenitors to deliver essential costimulatory signals might result in a state of induced unresponsiveness (anergy). DC propagated from normal mouse BM in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) + interleukin (IL)-4 ("mature" DC) are potent inducers of allogeneic T-cell activation. In contrast, costimulatory molecule-deficient DC progenitors (MHC class II$^+$, B7-1^{dim}, B7-2$^-$) grown in low concentrations of GM-CSF alone fail to stimulate in primary mixed leukocyte reactions (MLR) and induced donor-specific T-cell anergy (*15*). In this study, we have examined the relevance of these findings to organ transplantation by administering costimulatory molecule-deficient DC progenitors to normal mice that subsequently received vascularized cardiac allografts. The data show that pretreat-

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* Abbreviations: APC, antigen-presenting cell; BM, bone marrow; CTL, cytotoxic T lymphocyte; DC, dendritic cell; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; MLR, mixed leukocyte reactions; MST, median graft survival time.

ment with these donor-derived cells 1 week before transplant prolongs graft survival. Moreover, injection of DC progenitors is associated at the time of transplant with hyporesponsiveness of host T cells to donor alloantigens. In all cases, hyporesponsiveness to a third party was also observed. The eventual graft rejection may be ascribed to in vivo up-regulation of costimulatory molecule expression on donor-derived DC in the absence of exogenous immunosuppression.

MATERIALS AND METHODS

Animals. Ten- to 12-week old male C57BL/10J (B10; $H2^b$, $I\text{-}A^b$, $I\text{-}E^-$), C3H/HeJ (C3H; $H2^k$, $I\text{-}A^k$, $I\text{-}E^k$), and BALB/c ($H2^d$, $I\text{-}A^d$, $I\text{-}E^+$) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. They were maintained in a specific pathogen-free animal facility at the University of Pittsburgh Medical Center.

Propagation and purification of DC. BM cell suspensions were prepared in RPMI 1640 medium (Gibco, Grand Island, NY) using conventional procedures. The method for in vitro propagation of DC progenitors was modified (*16*) after that described initially by Inaba et al. (*17, 18*). Briefly, 2×10^6 cells were cultured in 24-well plates in RPMI 1640 complete medium supplemented with 10% v/v heat-inactivated fetal calf serum (Gibco) and 1000 U (4 ng)/ml recombinant mouse GM-CSF either alone or together with 1000 U/ml recombinant mouse IL-4 (both cytokines kindly provided by Dr. S.K. Narula, Schering-Plough Research Institute, Kenilworth, NJ). Nonadherent, low-buoyant density cells released spontaneously from proliferating cell clusters were recovered after 5–6 days of culture and resuspended in complete medium. They were layered onto 2-ml columns of metrizamide solution (14.5% w/v, grade 1, approximately 99% pure; Sigma Chemical Co., St. Louis, MO) sedimented at $600\times g$ at room temperature and recovered as described previously (*19*). The low-density interface (DC) population was washed twice before final resuspension in complete medium. Purity of the DC was verified by morphologic appearance of cytocentrifuge preparations (Geimsa staining), flow cytometric analysis, and immunocytochemical staining. An extensive panel of monoclonal antibodies was used, including those directed against mouse DC-restricted cell surface and intracellular antigens (*16, 20*). DC were also characterized by the presence or absence of phagocytic activity, using both carboxylated fluorescent latex microspheres (Fluoresbrite Carboxy YG, 0.5- to 3.0-μm diameter; Polysciences Ltd., Warrington, PA) (*21*) and opsonized (*16*) sheep red blood cells (Remel, Lenexa, KS). Only those DC preparations with purity >80% were used.

Flow cytometry. Cell surface immunophenotypic analysis was performed by cytofluorography using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Incubation with primary rat monoclonal antibodies, including rat anti-NLDC 145 (*22*), which is directed against a 205-kDa protein on mouse DC (*23*) (a gift from Dr. R.M. Steinman, The Rockefeller University, New York, NY), was followed by fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat Igs, as described (*16*). FITC-conjugated rat anti-mouse B7-1 (CD80) or B7-2 (CD86) (both PharMingen, San Diego, CA) were also used (*15*). MHC class II antigen expression was identified using biotin-conjugated mouse anti-mouse monoclonal antibodies (PharMingen) with FITC streptavidin (Jackson Immunoresearch Laboratories Inc., West Grove, PA) as the secondary reagent (*10, 16*). After staining, the cells were fixed in 1% paraformaldehyde in saline before analysis.

Heterotopic heart transplantation. The method for heterotopic heart transplantation was adapted from the rat procedure of Ono and Lindsey (*24*). The heart was transplanted into the abdomen with end-to-side anastomosis of aorta to aorta and pulmonary artery to vena cava. Rejection was defined by the cessation of cardiac contraction after daily palpation through the abdominal wall, and confirmed by histological examination. No immunosuppressive therapy was used. To test the influence of cultured cells on heart allograft survival, 2×10^6 cells were injected intravenously in 0.2 ml of medium via the lateral tail vein, 7 days before organ transplantation.

Mixed leukocyte reactions. Freshly isolated splenic T cells for use as responder cells were prepared as described previously (*16*). For primary MLR, 2×10^5 T cells were cultured with graded concentrations of γ-irradiated (20 Gy) allogeneic or syngeneic stimulator cells in 0.2 ml of RPMI 1640 complete medium and 10% fetal calf serum for 3 days in 96-well, round-bottom tissue culture plates in 5% CO_2 in air. For secondary MLR, primary cultures (2×10^6 T cells incubated with 10^6 stimulators in 24-well plates in 2 ml of culture medium) were harvested after 2 days, washed, and depleted of Ia^+ and nonviable cells, as described (*15*). The pretreated T cells were rested for 2 days, then restimulated with freshly isolated γ-irradiated spleen cells in a secondary, 3-day MLR in 96-well plates. For the final 18 hr of culture, 10 μl of [^{3}H]thymidine deoxyribose (1 μCi) were added to each well. Cells were harvested onto glass fiber disks using a multiple cell harvester. The degree of thymidine incorporation was determined in a liquid scintillation counter. Results were expressed as mean counts per minute ± 1SD.

Cell-mediated lymphocytotoxicity assay. Freshly isolated spleen cells from C3H mice were cultured with γ-irradiated (20 Gy) allogeneic (B10) spleen cells (ratio 2:1) for 6 days then used as effectors. The EL4 lymphoma cell line ($H2^b$; TIB39, ATCC, Rockville, MD) was used as a source of target cells. The R1.1 mouse lymphoma cell line ($H2^k$; TIB42, ATCC) and the P815 mouse mastocytoma cell line ($H2^d$; TIB64, ATCC) were used as syngeneic control and third-party targets, respectively. The target cells were labeled with 100 μCi of $Na_2{}^{51}Cr\ O_4$ (NEN, Wilmington, DE), washed, and plated at a concentration of 5×10^3 cells/well in 96-well round-bottom culture plates (Corning, Corning, NY). Serial, twofold dilutions of effector cells were added to give E:T ratios of 100:1, 50:1, 25:1, and 12.5:1 in a total volume of 200 μl/well. The percentage of specific ^{51}Cr release was determined after the plates had been incubated for 4 hr at 37°C in 10% CO_2 in air. An aliquot (100 μl) of supernatant was recovered from each well after centrifugation at 500g for 1 min. Maximum ^{51}Cr release was determined by osmotic lysis of the cells. The percent specific cytotoxicity was calculated using the following formula:

$$\%\ cytotoxicity = \frac{experimental\ (\text{cpm}) - spontaneous\ (\text{cpm})}{maximum\ (\text{cpm}) - spontaneous\ (\text{cpm})} \times 100$$

The results are expressed as mean % specific ^{51}Cr release ± 1SD in triplicate cultures.

Immunohistochemistry. Two-color immunofluorescence labeling of donor-derived cells in acetone-fixed cryostat sections of spleen was performed as described previously (*4*). Briefly, endogenous biotin was blocked with an avidin-biotin blocking reagent (Vector Laboratories, Inc., Burlinghame, CA), followed by incubation with nonfat dried milk (each for 20 min). After two washes with phosphate-buffered saline, the tissues were reacted for 45 min with biotinylated donor-specific anti-MHC class II mAb, or with isotype-matched biotinylated control Ig, then washed twice in phosphate-buffered saline. The primary antibody was then localized by incubation with streptavidin-conjugated Cy3.18 (Jackson Immunoresearch Laboratories) for 30 min. Cells expressing B7-1 or B7-2 molecules were identified by incubation (30 min) with specific FITC-conjugated rat anti-mouse monoclonal antibodies (PharMingen) or with irrelevant isotype-matched antibody (negative control). Donor class II single positive cells were stained red; B7-1 or B7-2 single positive cells were stained green. Cytospin preparations of cultured DC were used as additional positive or negative controls.

Statistics. Median graft survival times (MST) between groups were compared using the Kruskal-Wallis test. Pairwise comparisons were performed using the Wilcoxon sum rank test. P-values <0.05 were considered statistically significant.

RESULTS

Immunophenotypic analysis of GM-CSF- and GM-CSF+IL-4-stimulated cells. The surface phenotype of cells released after 5–6 days from proliferating aggregates in GM-CSF±IL-4-stimulated BM-derived cell cultures was characterized by flow cytometric analysis. Staining for cells of lymphoid lineage, including natural killer cells, was absent. As described previously, however, the floating cells stimulated with GM-CSF alone stained to variable degrees for surface antigens that are known to be associated with mouse DC (*15, 16*). These included CD45 (leukocyte common antigen), MHC class I, heat-stable antigen (J11D), CD54 (intercellular adhesion molecule-1), CD11b (MAC-1), and CD44 (nonpolymeric determinant of Pgp.1 glycoprotein). In addition, staining of low to moderate intensity was observed for the mouse DC-restricted cell surface markers NLDC-145 (interdigitating cells), 33D1 and CD11c (N418; β2-integrin), for the macrophage antigen F4/80, and for FcγRII (CD32) (data not shown). As shown in Figure 1, the GM-CSF-stimulated BM-derived cells from B10, C3H, and BALB/c mice also expressed moderate levels of cell surface MHC class II, but were B7-1^{dim} (data not shown) and B7-$2^{-/dim}$. Culture of cells in GM-CSF+IL-4 augmented the intensity of staining for each of these molecules (Fig. 1). The expression of NLDC 145, MHC class II, and especially B7-2 was markedly and consistently up-regulated on cells cultured for 6 to 10 days in the presence of both cytokines. The GM-CSF-stimulated MHC class II^+ B7-1^{dim} B7-$2^{-/dim}$ DC progenitors and the GM-CSF+IL-4-induced MHC class II^+ B7-1^+ B7-$2^{+/bright}$ populations are subsequently referred to herein as B7-2^- and B7-2^+ DC, respectively.

Allostimulatory activity of "mature" (B7-2$^+$) DC and DC progenitors (B7-2$^-$) and the induction of T-cell hyporesponsiveness. As reported previously (*15*), the GM-CSF+IL-4-stimulated B10 BM-derived DC 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 (data not shown). These B7-2^+ cells were considered to be "mature" DC. In contrast, the same numbers of GM-CSF-stimulated MHC class II^+ B7-1^- B7-2^- B10 BM-derived cells were very poor inducers of allogeneic T-cell activation and exhibited a comparatively low level of MLR stimulatory activity, similar to that of bulk B10 spleen cells. This was consistent with their very low expression of cell surface B7-2. The B7-2^- cells were therefore considered to be functionally "immature" DC. Allogeneic (C3H) T cells that were exposed initially for 2 days to B10 B7-2^- BM DC then repurified and rested for 2 days responded only weakly upon restimulation with B10 spleen cells (data not shown).

B7-2$^-$ BM-derived DC prolong cardiac allograft survival. We next determined whether B7-2^- BM-derived DC progenitors could influence vascularized cardiac allograft survival in the absence of host immunosuppression. Graft survival time in untreated C3H recipients of B10 hearts ranged from 8 to 13 days. Neither fresh allogeneic B10 BM cells nor B10 B7-2^+ BM-derived DC significantly affected graft survival. In the latter group, however, 10/17 (59%) of the hearts were rejected in accelerated fashion within 7 days. By contrast, 2×10^6 B7-2^- B10 BM-derived DC given intravenously 7 days before heterotopic heart transplantation prolonged the MST of B10 cardiac allografts in C3H mice from 9.5 (untreated recipients) to 22 days (Table 1). Interestingly, third-party

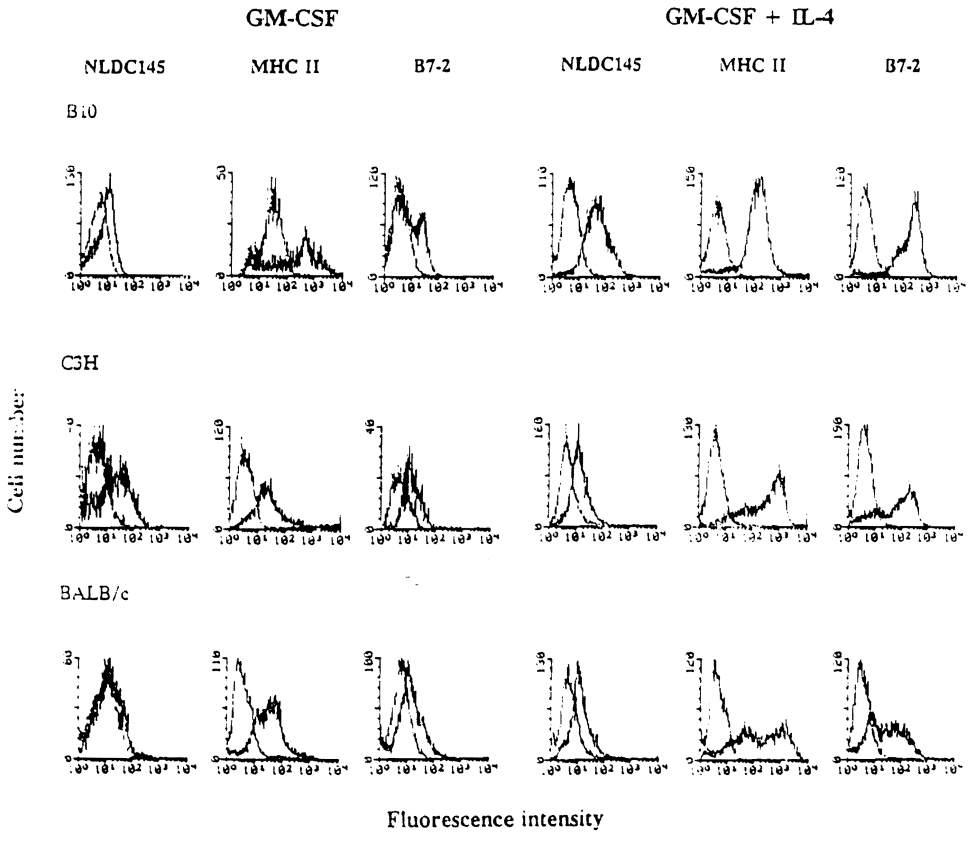

FIGURE 1. FACScan analysis of NLDC-145, MHC class II, and B7-2 expression on 6-day GM-CSF and 6-day GM-CSF+IL-4-stimulated B10, C3H, or BALB/c BM-derived DC. Further details are provided in *Materials and Methods* or have been described elsewhere (*15*). The appropriate Ig isotype subclass control is always to the left of the specific antibody profile. The results are representative of three separate experiments.

TABLE 1. Influence of donor-specific GM-CSF-stimulated DC progenitors (B7-2$^-$) on B10 cardiac allograft survival in C3H mice[a]

Group	Cells injected (day −7)	n	Graft survival times (days)	Mean±1SD	MST
A	None (media control)	8	8 (×3), 12, 13 (×2), 9, 10	10.1±2.2	9.5
B	Fresh B10 bone marrow cells (allogeneic)	4	12 (×4)	12	12
Cultured cells					
C	B10 (B7-2$^+$) (allogeneic)	17	4 (×5), 5 (×3), 7 (×2), 8 (×2), 9, 10, 14 (×2), 15	7.5±4.1	7[b]
D	B10 (B7-2$^-$) (allogeneic)	15	7, 19 (×4), 20, 22 (×2), 23, 26, 27, 29, 30, 35, 67	23.3±5.5	22[c,d]
E	C3H (B7-2$^-$) (syngeneic)	4	12 (×2), 13 (×2)	12.5±0.6	12.5
F	BALB/c (B7-2$^-$) (third party)	9	7, 12, 16 (×2), 17 (×3), 19, 20	15.7±3.9	16.5

[a] Cells (2×10^6 i.v.) were injected 7 days before heterotopic (B10→C3H) heart transplantation. MSTs were compared using the Kruskal-Wallis test. Pairwise comparison was done using the Wilcoxon sum rank test.
[b] $P<0.01$ compared with groups D and F.
[c] $P<0.003$ compared with groups C and E.
[d] $P<0.01$ compared with group F.

(BALB/c) B7-2$^-$ BM-derived DC also prolonged B10 heart graft survival, although the MST (16.5 days) was significantly shorter ($P<0.01$) than that achieved with donor-specific B7-2$^-$ DC.

MLR and cytotoxic T lymphocyte assays. To gain further insight into anti-donor immune responsiveness of C3H recipients of B7-2$^-$ or B7-2$^+$ B10 BM-derived DC, 2×10^6 cells were injected intravenously, and 7 days later (the time of heart transplant), MLR and cytotoxic T lymphocyte (CTL) assays were performed using recipient T cells as responder/effector cells. Figure 2 shows the MLR responses obtained against allogeneic (B10; H2^b), syngeneic (C3H; H2^k), and third-party (BALB/c; H2^d) stimulator cells at various S:R cell ratios. T cells from C3H mice injected with B10 B7-2$^+$ DC showed marked proliferative responses to donor, a much milder response to third-party cells, and no reactivity against syngeneic cells. By contrast, the animals that received B7-2$^-$ B10 DC progenitors showed very little evidence of MLR responses to donor and no reaction against third-party stimulators. A similar pattern of anti-donor reactivity was obtained using CTL assays (Fig. 3). T cells from mice injected 7 days earlier with 2×10^6 B10 B7-2$^+$ DC exhibited much higher CTL responses to donor targets than animals given B7-2$^-$ cells. A moderate CTL response to third-party targets was found in the former, but not in the latter, group of animals. The third-party response suggested a degree of antigenic cross-reactivity between B10 (H2^{b+}) and BALB/c (H2^{d+}) cells or between the target cell lines expressing the respective histocompatibility antigens.

Expression of B7-1 and B7-2 molecules on donor-derived cells within recipient lymphoid tissue. To determine whether the B7-2$^-$ BM-derived DC progenitors expressed cell surface B7 family molecules following their systemic administration, 2×10^6 B10 B7-2$^-$ cells were injected intravenously into naive C3H recipients. One to 7 days later, the animals were killed. Spleens were removed and analyzed by two-color immunohistochemical staining for the expression of B7-1 and B7-2 on donor-derived (H2^{b+}; I-A$^+$) cells. As shown in Figure 4, I-A^{b+} cells that also expressed B7-1 or B7-2 could be detected in T-dependent areas within 7 days of B7-2$^-$ cell injection. Thus, although B10 B7-2$^-$ DC induced only low levels of T-cell priming and prolonged B10 heart graft survival, B7 family molecule expression could nevertheless be detected on these cells in normal nonimmunosuppressed recipients within 1 week of their administration.

DISCUSSION

The present findings show, for the first time, that pretreatment of mice with MHC class II$^+$ costimulatory molecule (B7 family) "deficient" DC progenitors can prolong vascularized organ allograft survival. The systemic administration of these donor-BM-derived APC 1 week before cardiac transplantation resulted in temporary prolongation of graft survival, in the absence of immunosuppressive drug therapy. MST was prolonged significantly from 9.5 days (untreated controls) to 22 days; a less marked but statistically significant prolongation of heart allograft survival was also obtained by injection of third-party DC progenitors. In addition, ex vivo analysis of host T-cell responses to donor alloantigens revealed only weak MLR and CTL function in animals pretreated with DC progenitors compared with those primed with "mature" (B7-1$^+$, B7-2$^+$) DC. The results are consistent with the capacity of the BM-derived DC progenitors to induce T-cell hyporesponsiveness to alloantigen in vitro. They are also in agreement with our recent finding (*25*) that, using a similar cell dose and treatment schedule, DC progenitors prolong the survival of pancreatic islet allografts.

Considerable insight has recently been gained into the role of costimulation in T-lymphocyte activation. Costimulatory signals delivered to T cells by APC are neither antigen specific nor MHC restricted, but they determine the outcome of T cell receptor signaling because they mediate cytokine secretion. Co-ligation of the T-cell CD28/CTLA-4 receptors (an essential stimulus of T-cell activation) (*26*) by B7 family molecules (*27-29*) results in IL-2 production and T-cell proliferation (*26*, *28*, *29*). Although DC express several other important intercellular adhesion and costimulatory molecules, including CD11a, CD40, CD44, CD48/58, and CD54, B7-2 (CD86) appears to be the major CD28/CTLA-4 ligand and accounts for the costimulation provided by this pathway during in vitro T-cell responses. The early and sustained expression of B7-2 mRNA (within 8 hr) (before maximal B7-1 expression) in activated murine B cells (*30*) suggests that it may provide a critical signal involved in the decision between activation or inactivation of T cells within 24 hr of stimula-

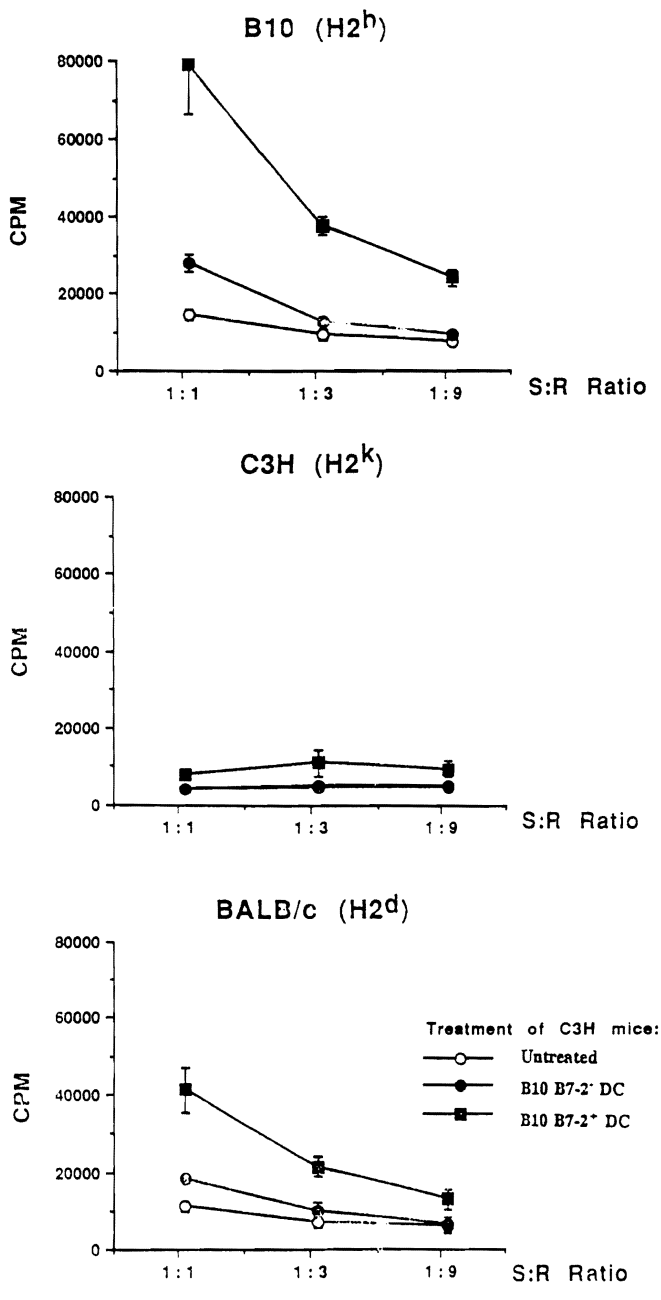

FIGURE 2. Mixed leukocyte responses of splenic T cells from normal C3H mice injected intravenously 7 days previously with either 2×10^6 (●) B10 BM-derived B7-2$^-$ DC progenitors or (■) B7-2$^+$ DC. The C3H T cells were stimulated for 3 days with either B10 (allogeneic), C3H (syngeneic), or BALB/c (third party) spleen cells at various S:R ratios. Results are mean counts per minute ± 1SD and are representative of three separate experiments.

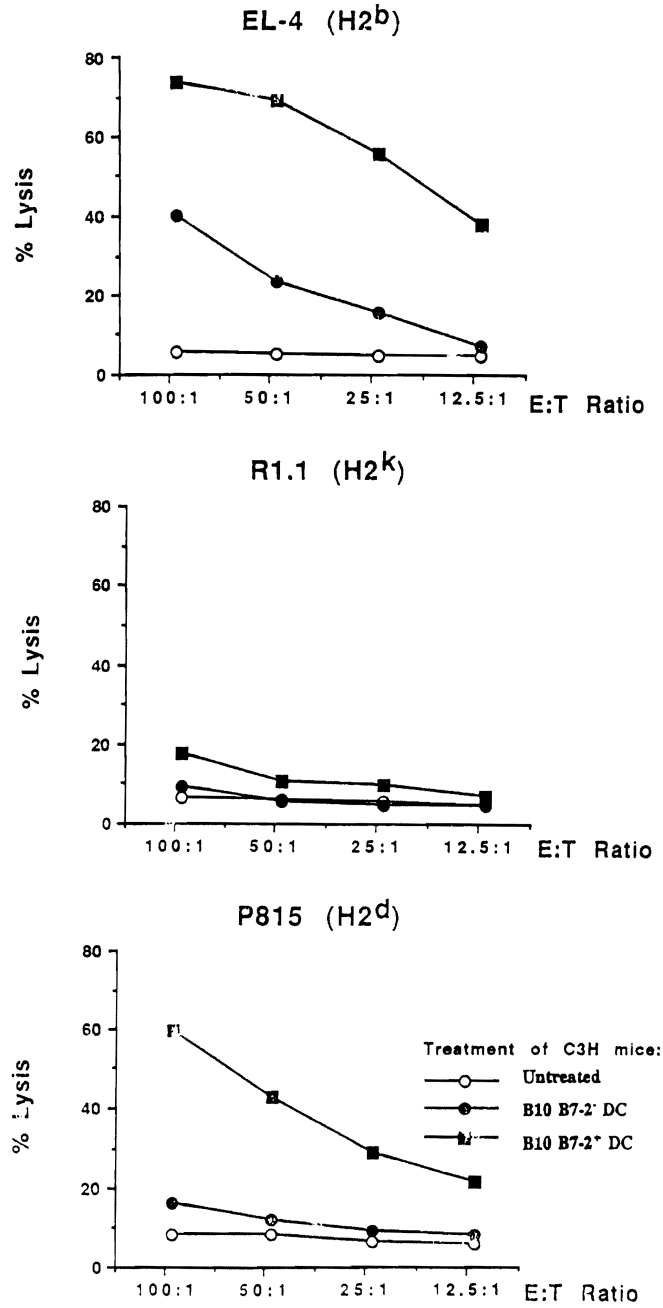

FIGURE 3. CTL responses of splenic T cells from normal C3H (H2^k) mice injected intravenously 7 days previously with either 2×10^6 (●) B10 (H2^b) BM-derived B7-2$^-$ DC progenitors or (■) B7-2$^+$ DC. Freshly isolated C3H spleen cells were cultured for 6 days with γ-irradiated B10 spleen cells, as described in *Materials and Methods*, then used as effectors against ^{51}Cr-labeled allogeneic EL-4 (H2^b), syngeneic R1.1 (H2^k), or third-party P815 (H2^d) target cells. The results are expressed as mean % specific lysis in triplicate cultures.

tion (*31*, *32*). Moreover, APC from B7-1 "knockout" mice that express B7-2 are competent APC (*30*).

MHC class II$^+$ DC progenitors that are B7-2$^-$ may (as is the case with inhibition of B7-2 expression [*27*]) allow T cell receptor-mediated signaling events to occur, but inhibit the distinct costimulatory signal(s) that is 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 gene transcription factors NF-AT or NF-kB (*33*).

In principle, the induction of irreversible T-cell anergy to donor-specific alloantigens in vivo (tolerance) would eliminate the need for potentially toxic, nonspecific immunosuppressive agents, which currently constitute first choice antirejection therapy. Thus, there has been considerable interest in evidence that blockade of the B7-CD28 T-cell costimula-

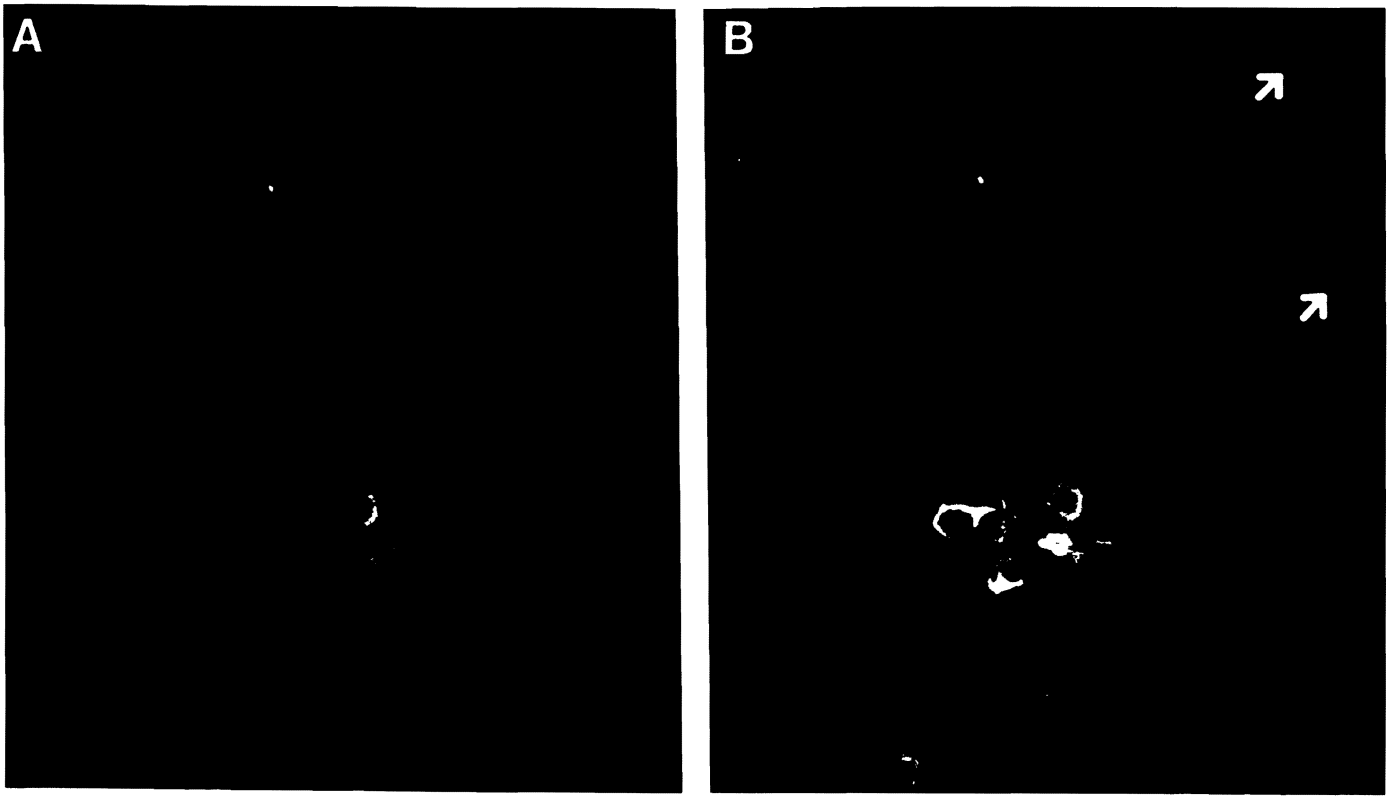

FIGURE 4. Two color immunofluorescence labeling of B7-2 (CD86) on donor-derived DC (I-A^{b+}) within the T-dependent area of allogeneic spleen 7 days after the injection of GM-CSF-stimulated DC progenitors (B7-2^-). (A) B7-2^+ cells are stained green (FITC-conjugated secondary mAb) and, in addition, (B) donor MHC class II^+ cells (I-A^{b+}) are stained red (steptavidin-conjugated Cy 3.18). (B) Both double-positive cells and less brightly staining (B7-2^-) donor cells (I-A^{b+}; arrows) are visible (magnification ×250).

tory pathway may be both necessary and sufficient to induce antigen-specific T-cell anergy (*34*). Combination of anti-B7 mAb and cyclosporine induces alloantigen-specific anergy in vitro (*35*). CTLA4-Ig, a fusion protein that blocks the CD28-mediated costimulatory signal, inhibits T-cell proliferation in allogeneic MLR (*27, 41*), and induces hyporesponsiveness to alloantigen in vitro (*11*). Moreover, it prolongs the survival of MHC-mismatched cardiac (*36, 37*) and renal (*38*) allografts in rats and induces donor-specific tolerance to skin grafts in cardiac-allografted mice (*39*). Propagation of B7-2^- "immature" DC with the capacity to induce hyporesponsiveness to donor alloantigens has significant in vivo implications. Conceivably, the tolerogenicity of the transplanted mouse liver, a potential hematopoietic organ (*40, 41*), may be linked, at least in part, to the release of potentially T cell-anergizing DC progenitors from the graft. This is likely to occur under the influence of locally produced GM-CSF (*42*) and possibly other DC growth-promoting cytokines, such as c kit-ligand, IL-3, and Flt3 ligand. Others have suggested that in immunosuppressed recipients, vascularized allogeneic bone grafts, with their content of DC progenitors, may induce both substantial chimerism and perhaps tolerance at the level of the "immature" DC-host T-cell interaction (*43*). The failure of the B7-2^- DC progenitors in this study to induce indefinite cardiac allograft survival may reflect instability of specific T-cell hyporesponsiveness in recipients of DC progenitors that have not received any form of immunosuppressive therapy. Graft rejection may also reflect the eventual in vivo up-regulation of at least one set of costimulatory (B7) molecules observed on some of these donor-derived cells within a week of their injection. Regulation of the expression of other molecules, e.g., CD40 or CD54 (intercellular adhesion molecule-1) is also likely to be important. It seems clear that considerations of the cellular therapy of allograft rejection using "immature" donor-derived DC progenitors should include further analysis of the influence of cell dosage, frequency of injection, and temporal relationship between cell injection and organ transplantation. The use of adjunctive immunosuppression with drugs or monoclonal antibodies should also be explored. Alternatively, molecular (gene therapy) approaches geared to inhibit or block costimulatory molecule expression on donor APC offer exciting possibilities.

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