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Growth of Donor-derived Dendritic Cells from the Bone Marrow of Murine Liver Allograft Recipients in Response to Granulocyte/Macrophage Colony-stimulating Factor

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Summary

Allografts of the liver, which has a comparatively heavy leukocyte content compared with other vascularized organs, are accepted permanently across major histocompatibility complex barriers in many murine strain combinations without immunosuppressive therapy. It has been postulated that this inherent tolerogenicity of the liver may be a consequence of the migration and perpetuation within host lymphoid tissues of potentially tolerogenic donor-derived ("chimeric") leukocytes, in particular, the precursors of chimeric dendritic cells (DC). In this study, we have used granulocyte/macrophage colony-stimulating factor to induce the propagation of progenitors that give rise to DC (CD45⁺, CD11c⁺, 33D1⁺, nonlymphoid dendritic cell 145⁺, major histocompatibility complex class II⁺, B7-1⁺) in liquid cultures of murine bone marrow cells. Using this technique, together with immunocytochemical and molecular methods, we show that, in addition to cells expressing female host (C3H) phenotype (H-2K^{b+}; I-E⁺; Y chromosome⁻), a minor population of male donor (B10)-derived cells (H-2K^{b+}; I-A⁺; Y chromosome⁺) can also be grown in 10-d DC cultures from the bone marrow of liver allograft recipients 14 d after transplant. Highly purified nonlymphoid dendritic cell 145⁺ DC sorted from these bone marrow-derived cell cultures were shown to comprise ~1-10% cells of donor origin (Y chromosome⁺) by polymerase chain reaction analysis. In addition, sorted DC stimulated naive, recipient strain T lymphocytes in primary mixed leukocyte cultures. Evidence was also obtained for the growth of donor-derived cells from the spleen but not the thymus. In contrast, donor cells could not be propagated from the bone marrow or other lymphoid tissues of nonimmunosuppressed C3H mice rejecting cardiac allografts from the same donor strain (B10). These findings provide a basis for the establishment and perpetuation of cell chimerism after organ transplantation.

An unresolved enigma with the recent demonstration of persistent low-level donor leukocyte chimerism after organ transplantation (1-4) has been the means by which the chimeric cells can be perpetuated up to many years (2) postoperatively. Both in rodents (5-7) and in humans (8), dendritic cells (DC),¹ which have well-recognized *in vivo* migratory ability (9-12), feature prominently in immunohistochemical analyses of this multilineage chimerism in long-term successful organ recipients. For some time, it has been recognized that DC in lymphoid and other organs arise from proliferating bone marrow-derived precursors (13-15). Only recently, how-

ever, *in vitro* systems have been developed for inducing the growth of these cells from precursors present in either the blood (16) or bone marrow (17). Similar methods have confirmed the presence of DC progenitors within the spleen (18) or liver (19). In each of these systems, liquid culture has been used to generate DC under the aegis of GM-CSF. This approach has permitted extensive characterization of the progeny DC, including their structural features, motility, immunophenotype, allostimulatory activity, and *in vivo* homing ability. With respect to murine liver-derived DC progenitors, it has been shown that their injection into nonimmunosuppressed allogeneic recipients gives rise to strongly MHC class II-positive cells that persist for months within T-dependent areas of secondary lymphoid tissue (20).

In mice, liver but not other organ allografts are accepted

¹ Abbreviations used in this paper: DC, dendritic cells; NPC, liver nonparenchymal cells; OLTx, orthotopic liver transplantation.

permanently and with donor specificity between many strain combinations, without the requirement for immunosuppression (6). Here we present evidence that liver allografts export DC precursors that can be propagated in GM-CSF from the bone marrow or spleen of unmodified recipients. This novel finding, together with failure to propagate donor cells from the lymphoid tissues of nonimmunosuppressed mice rejecting cardiac allografts from the same donor strain, is congruent with the possibility that bidirectional leukocyte migration and donor cell chimerism play key roles in organ graft acceptance and acquired transplantation tolerance.

Materials and Methods

Animals. 10–12-wk-old male C57BL/10J mice (B10; H-2^b, I-A^b, I-E⁻) and C3H/HeJ (C3H; H-2^k, I-A^k, I-E^k) mice of both sexes were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center (Pittsburgh, PA).

Liver and Heart Transplantation. Orthotopic liver transplantation (OLTx) was performed in a mouse strain combination (B10 to C3H; MHC class I, II and multiple non-MHC antigen disparities) with a high liver acceptance rate (6), using techniques described previously (21) with minor modifications. Briefly, after clamping the artery and portal vein, the donor liver was flushed thoroughly via the portal vein with cold lactated Ringer's solution. A suprahepatic vena cava anastomosis was completed, with a 10-0 running suture. The cuff technique was used for anastomosis of both the intrahepatic vena cava and the portal vein. No attempt was made to reconstruct the graft arterial supply. The bile ducts were anastomosed by inserting a polyethylene tube into both. Blood lost during the operation was replaced with lactated Ringer's solution. The method for heterotopic heart transplantation was adapted from the rat procedure of Ono and Lindsey (22). 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 it was confirmed by histological examination. No immunosuppressive therapy was used in either procedure, and the animals (three per group in each experiment) were killed 14 d after OLTx or 8 d after heart transplantation.

Isolation of Leukocytes from Tissues. Before harvesting of organs from transplanted animals, whole body perfusion was performed to minimize blood contamination of tissues. 30 ml HBSS (GIBCO BRL, Gaithersburg, MD) was infused over a 3-min period via the inferior vena cava. Bone marrow cell suspensions and mononuclear cells from the spleen or thymus were prepared in RPMI 1640 (GIBCO BRL) using conventional methods. Liver nonparenchymal cells (NPC) (<5% hepatocyte contamination) were isolated as described in detail elsewhere (19). Briefly, the liver was perfused *in situ* via the portal vein with collagenase solution (type IV, 1 mg/ml in HBSS; Sigma Chemical Co., St. Louis, MO). It was then removed, diced, and further digested with collagenase *in vitro*. The washed cells were then centrifuged on Percoll (Sigma Chemical Co.) gradients, and the leukocyte-rich layer was harvested as described (19).

Culture of Tissue-derived Cells with GM-CSF. 1–2 × 10⁶ cells obtained from the bone marrow, spleen, thymus, or liver were cultured per well in 24-well plates in 2 ml RPMI 1640, supplemented with 10% FCS and 0.4 ng/ml mouse rGM-CSF (R&D Systems,

Minneapolis, MN). In some experiments, bone marrow-derived cells were cultured in GM-CSF (4 ng/ml) plus IL-4 (1,000 U/ml) (each cytokine was from Schering-Plough, Kenilworth, NJ) in an effort to maximize the development of their allostimulatory activity (23). The procedure used was similar to that described initially by Inaba et al. (17) for the propagation of large numbers of DC from mouse bone marrow. The medium containing GM-CSF (or GM-CSF plus IL-4) was refreshed every 2 d; after gentle swirling of the plates, half of the old medium was aspirated and an equivalent volume of fresh cytokine-supplemented medium added. This method was implemented to deplete nonadherent granulocytes without dislodging clusters of developing DC that attached loosely to a monolayer of firmly plastic-adherent macrophages. Morphological and phenotypic analyses of typical, single, nonadherent mononuclear cells released spontaneously from the clusters were performed after 10 d.

Staining of Cell Surface and Intracellular Antigens. Cell surface staining was analyzed by cytofluorography using a FACScan[®] flow cytometer (Becton Dickinson & Co., Mountain View, CA). Staining with primary hamster or rat 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 (18, 19). Donor and recipient 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 Laboratories, Inc., 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 DC-restricted granule antigen were tested on cytospin preparations as described (17, 18). 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 Corp., Indianapolis, IN) or peroxidase-conjugated anti-rat Ig, respectively. The nuclei were counterstained with hematoxylin.

Detection of Donor Male (Y) Chromosome in Cell Cultures by PCR Analysis. DNA was prepared from freshly isolated and 10-d GM-CSF-stimulated cells from both normal male B10 and female C3H mice and from female C3H mice 14 or 8 d, respectively, after liver or heart transplantation from male B10 donors. The presence of male donor cells was determined as previously described for human analysis (24) by PCR amplification of the SRY region of the mouse Y chromosome. Primers (5'-CAGCCCTACAGCCACAT-3' and 5'-CCACTCCTCTGTGACACTTT-3') were chosen from the p4.2.2 sequence (25). 1-μg aliquots of DNA were amplified with 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s in buffer consisting of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.2 mM deoxynucleotides, 0.1% gelatin, 0.2 μM primers, and 2.5 U of Taq polymerase (Perkin-Elmer Corp., Norwalk, CT). The products were separated in a 1% agarose gel, transferred to nylon membrane, and hybridized with an identical purified and radiolabeled ³²P PCR product from male mouse DNA. Single-copy sensitivity and male specificity were verified with control samples consisting of serial dilutions of male mouse DNA into that of female mouse DNA.

Sorting of DC. Before staining with mAb, cultured bone marrow-derived cells were incubated with 10% goat serum (Vector Laboratories, Inc., Burlingame, CA) in HBSS (GIBCO BRL) at 4°C for 30 min to eliminate nonspecific binding. The cells were then washed once in HBSS containing 0.1% BSA (Sigma Chemical Co.) and resuspended to a concentration of 40 × 10⁶ ml in the same buffer. A saturating concentration of nonlymphoid dendritic cells [NLDC] 145 mAb (a gift from Dr. R. M. Steinman,

The Rockefeller University, New York) or rat IgG2a isotype control (Sigma Chemical Co.) was added, and cells were incubated for 45 min at 4°C. After two washes in HBSS, the cells were stained with FITC-conjugated goat anti-rat IgG (Sigma Chemical Co.) under the same conditions. They were then washed and resuspended in complete RPMI 1640 medium supplemented with 2% FCS (GIBCO BRL) for cell sorting. NLDC 145⁺ and NLDC 145⁻ cells were sorted into distinct cell populations based on forward scatter, side scatter, and FITC fluorescence intensity using a FAC-Star Plus[®] cell sorter (Becton Dickinson & Co.). Cells were excited with a 5-mW argon/HeNe laser (Coherent Inc., Palo Alto, CA) attaining purities of both populations >90%.

Mixed Leukocyte Cultures. To test the allostimulatory activity of cultured, bone marrow-derived cells, one-way mixed leukocyte cultures (2×10^5 responder cells per well in 96-well, round-bottom microculture plates) were performed. Various numbers of γ -irradiated (20 Gy) stimulator cells propagated with GM-CSF plus IL-4 from donor (B10) or recipient (C3H) strain animals or from the bone marrow of liver allograft recipients were added. As an aid to determining the incidence of donor-derived allostimulatory cells propagated from graft recipients, "artificial" mixtures of cultured donor (1–10%) and recipient strain bone marrow-derived cells were also used as stimulators. C3H or B10 spleen cells were used as responders, which were T cell enriched by sequential removal of plastic adherent cells (1 h at 37°C) and by passage (1 h) through a nylon wool column. Cultures were maintained in RPMI 1640 complete medium supplemented with 10% heat-inactivated FCS for 72 h in 5% CO₂ in air; for the final 18 h, 10 μ l [³H]TdR (1 μ Ci) was added to each well. Cells 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.

Results

Propagation of DC from Normal Mouse Bone Marrow. In prior studies, aggregates of growing DC progenitors were described in cultures established from mouse liver or spleen cell suspensions (18, 19) using procedures modified after Inaba and co-workers (16, 17). Similar techniques were adopted to propagate DC progenitors from the bone marrow of B10 and C3H mice. At 2 and 4 d of culture, non- or loosely adherent granulocytes were removed, without dislodging clusters of developing DC that were attached to firmly plastic-adherent macrophages. After day 4, granulocytes were no longer significant contaminants, and the cultures were maintained routinely for 10 d. Numerous dendritic-shaped cells appeared to be released from the clusters. These floating cells exhibited typical dendritic projections and other structural features of DC (17, 19, 26) upon light microscopic examination.

Immunophenotypic Analysis of GM-CSF-stimulated Bone Marrow-derived Cells. To ascertain the surface phenotype of cells released from proliferating aggregates, flow cytometric analysis was performed after 10 d of culture in GM-CSF. Staining for cells of lymphoid lineage (CD3⁺, CD4⁺, CD8⁺, B220⁺) including NK cells (NK1.1⁺) was absent. The floating cells in 10-d bone marrow-derived cultures expressed surface antigens that are known to be associated with murine DC. These included CD45 (leukocyte common antigen), MHC class I, heat-stable antigen (J11D), CD54 (intercellular

adhesion molecule-1), CD11b (membrane attack complex-1), and CD44 (nonpolymeric determinant of Pgp.1 glycoprotein). In addition, staining of moderate intensity was observed for the murine DC-restricted markers NLDC 145 (interdigitating cells), 33D1, and CD11c (N418; β 2-integrin); for the macrophage antigen F4/80; and for Fc γ RII, as described previously (17) for bone marrow-derived DC. The cells also expressed moderate levels of MHC class II and of the costimulatory molecule and CD28 ligand, B7-1. The cells were negative, however, for cell surface expression of the CD28 coligand B7-2. Expression of the low affinity IL-2 receptor (CD25) could also not be demonstrated (data not shown).

Cytospins were prepared to further characterize the released DC. By day 10, the majority of the cells were dendritic by Giemsa stain and expressed high levels of MHC class II. Granulocytes were rare. The majority of the cells strongly expressed the DC-restricted granule antigen 2A1 (17, 18). Similar results were obtained whether the cultures were established from cells obtained from the bone marrow of either B10 or C3H mice.

Detection of Cells Expressing Donor Phenotype in Freshly Isolated Bone Marrow from Liver Allograft Recipients. C3H mice accept livers from B10 donors spontaneously, with graft survival times consistently >100 d (6). To identify cells expressing donor phenotype in freshly isolated bone marrow cell suspensions obtained from nonimmunosuppressed C3H mice 14 d after OLTx from B10 donors, mAbs directed against donor (H-2^b) and recipient (H-2^k) MHC class I antigens were used. Typically, as shown in Fig. 1 (left), the incidence of cells expressing donor MHC class I determined by flow cytometry in allografted mice was very low (\sim 1%), whereas that of recipient was, as expected, consistently very high (>84%).

Cells Expressing Donor Phenotype in Bone Marrow DC Cultures from Liver Allograft Recipients. We next determined whether DC expressing donor cell surface phenotype could be propagated using GM-CSF from postulated small numbers of progenitor cells in freshly isolated bone marrow of unmodified liver allograft recipients. As shown in Fig. 1 (right), both cells expressing recipient (H-2K^{k+}) and those expressing donor MHC class I (H-2K^{b+}) could be identified by cytofluorometry in 10-d cultures propagated using the same methods as for DC from normal bone marrow. Staining of cytopins for recipient and donor MHC class II products confirmed the presence of small numbers of donor (I-A^{b+}) cells (Fig. 2). In several repeat experiments using pooled bone marrow cells from groups of three allograft recipients, the incidence of donor MHC class II⁺ cells in 10-d GM-CSF-stimulated cultures ranged from 5 to 17%.

Detection of Donor Y Chromosome in GM-CSF-stimulated Cultures from Liver Allograft Recipients. The apparent incidence of donor-derived DC in GM-CSF-stimulated bone marrow cell cultures was comparatively low. Therefore, to eliminate the possibility of false positive results due to minimal antibody cross-reactivity or to the uptake of donor MHC products by recipient APC, PCR analysis for donor Y chromosome was performed on cells cultured from female (C3H) recipients of male (B10) liver grafts killed 14 d after transplant. Detection of Y chromosome (Fig. 3) confirmed the

BONE MARROW: MHC CLASS I

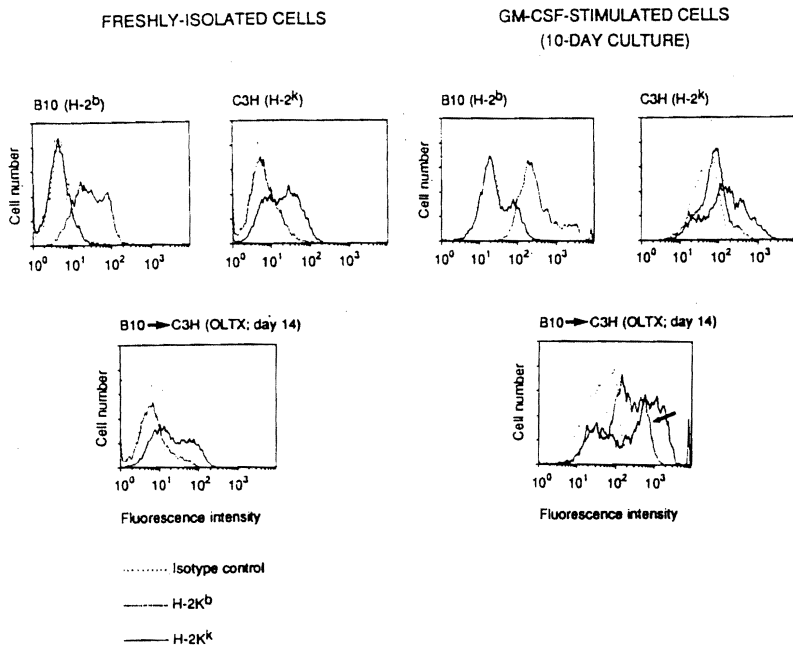


Figure 1. Flow cytometric analyses (MHC class I staining) of freshly isolated (*left*) and GM-CSF-stimulated bone marrow DC (*right*) from normal B10 (H-2^b), normal C3H (H-2^k), and unmodified B10 → C3H orthotopic liver allograft recipients 14 d after transplant. Donor strain (B10) and recipient strain cells (C3H) are identified by staining for H-2K^b and H-2K^k, respectively. Cultured cells were maintained for 10 d before analysis. Note the coexistence of cells expressing donor class I (H-2K^b line; arrowed) and recipient class I in the DC cultures propagated from transplanted mice (*bottom right*). The result is representative of three separate experiments.

growth of donor-derived cells in the 10-d bone marrow cell cultures and reinforced the immunocytochemical observations. Y chromosome signal was also detected in 10-d spleen cell cultures and was also increased in magnitude compared with freshly isolated cells. The signal, however, was substantially lower compared with bone marrow-derived cells (Fig. 3), indicating that comparatively few donor-derived, GM-CSF-responsive DC precursors had reached the spleen. A diminished signal for donor (male) cells in 10-d GM-CSF-stimulated liver-derived cell populations (Fig. 3) may reflect the deliberate physical removal and also the death of contaminating male hepatocytes (<5% at the initiation of the cultures). In addition, in situ replacement of donor (male) with host (female) GM-CSF-responsive cells within the liver by day 14

after transplant (5, 6), when the cells were freshly isolated, may contribute to the relative reduction in the donor signal from cultured liver-derived cells on day 10. In contrast to bone marrow and spleen, and despite histological findings (5) and evidence for chimerism in freshly isolated thymus cell suspensions, no signal for donor-derived cells could be detected in 10-d GM-CSF-stimulated cultures of thymocytes from liver allograft recipients. This suggested that, although a site in which chimeric cells are detected after OLTx, the thymus may not be a destination for adequate numbers of donor-derived immature DC progenitors.

Evidence that the Donor-derived Cells are DC. We next sought direct evidence that the donor-derived cells propagated from the bone marrow of liver allograft recipients were DC. Sorting

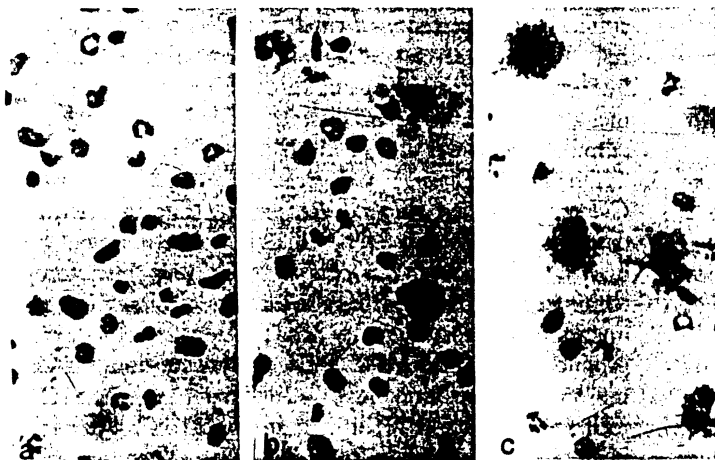


Figure 2. Coexistence of cells expressing donor (H-2^b; I-A^b) and recipient strain (H-2^k; I-E^k) MHC class II phenotype in 10-d cultures of GM-CSF-stimulated cells propagated from the bone marrow of liver-allografted [B10 (H-2^b) → C3H (H-2^k)] mice killed 14 d after transplant. Staining was performed using the appropriate biotinylated mouse anti-mouse mAb, followed by streptavidin biotin peroxidase complex, as described (19). (a) Ig isotype control; (b) evidence of occasional donor (I-A^b+) cells; as well as (c) DC expressing recipient phenotype (I-E^k+) cells. Counterstained with hematoxylin. ×250.

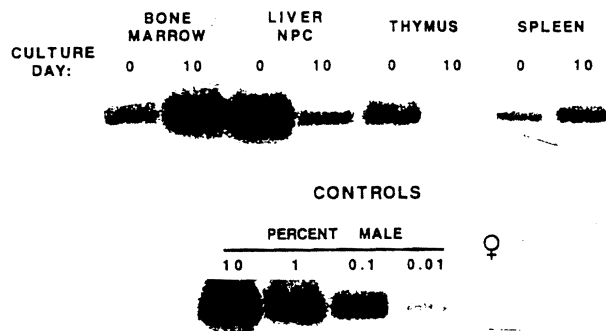


Figure 3. Detection of the donor Y chromosome in cells cultured from female recipients of male livers killed 14 d after OLTx. PCR analysis was performed on freshly isolated (day 0) and day 10 GM-CSF-stimulated bone marrow, liver NPC, spleen, or thymus cultures. There is convincing evidence of growth of donor-derived cells from the bone marrow and also the spleen, while no male cells appear to have propagated from the thymus. Survival of donor liver-derived cells is also evident (for discussion, see text). Similar results were obtained in three separate experiments using cells pooled from three transplanted mice.

of donor-positive cells was considered, but the anticipated yield of cells was calculated to be too low for subsequent functional analysis. Instead, 10-d GM-CSF-stimulated, bone marrow-derived cells were harvested, and NLDC 145⁺ cells were sorted (at least 90% purity by morphologic and FACScan[®] analysis; Fig. 4, a and b) and then investigated for the presence of donor Y chromosome. As shown in Fig. 4 c, the results of PCR analyses demonstrated convincingly that the highly purified DC population comprised ~1–10% Y chromosome-positive (donor-derived) cells. Further evidence for the presence of donor-derived DC was obtained by testing the allostimulatory activity of sorted, NLDC 145⁺ GM-CSF plus IL-4-stimulated, bone marrow-derived cells in primary MLR. As shown in Fig. 5 A, the purified NLDC 145⁺ population propagated from C3H recipients of B10 allografts strongly stimulated B10 responders but also stimulated a response in recipient strain T cells (Fig. 5 B). The extent of stimulation ($P < 0.01$ compared with negatively sorted cells or syngeneic DC) was similar to that achieved with “artificial mixtures” containing 1% donor strain DC.

Failure to Propagate Donor-derived Cells from Bone Marrow of Mice Rejecting Heart Allografts. In contrast to liver allograft recipients, nonimmunosuppressed C3H mice reject heart allografts from the same donor strain (B10) within 8 d (6). PCR analysis for donor Y chromosome was performed on 10-d GM-CSF-stimulated cultures of bone marrow cells harvested from heterotopic cardiac allograft recipients 8 d after transplant. In contrast to the results obtained from liver graft recipients, no evidence was obtained for the propagation of donor-derived cells from the bone marrow of animals rejecting their cardiac grafts (Fig. 6), despite evidence of chimeric cells in freshly isolated bone marrow. It thus appears that these heart-derived cells, in contrast to those detected in fresh bone marrow of liver allograft recipients, do not comprise sufficient numbers of GM-CSF-responsive progenitors to allow growth

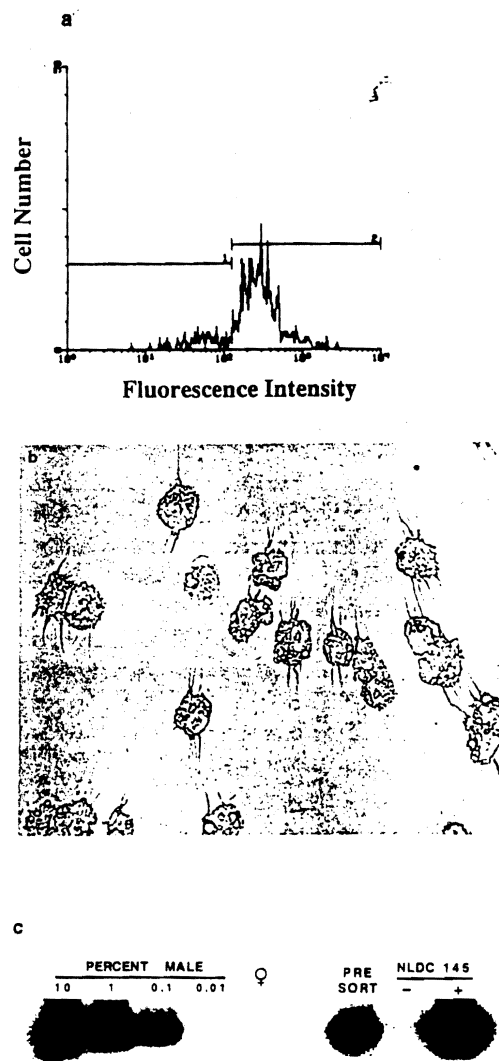


Figure 4. Demonstration that donor-derived cells in 10-d GM-CSF-stimulated bone marrow cell cultures from liver-allografted mice are DC. (a) FACScan[®] profile of sorted NLDC 145⁺ cells from GM-CSF-stimulated bone marrow (day 10) reanalyzed from purity of the sorted population. Horizontal bars indicate (1) limits of range of negatively sorted cells; and (2) sorted NLDC 145⁺ cells (>90% purity). (b) Cytospin preparation showing the dendritic morphology of the sorted NLDC 145⁺ population $\times 250$. (c) PCR analysis of pre- and postsorted cells, showing the presence of donor-derived Y chromosome in the NLDC 145⁺ fraction.

of donor-derived cells (as opposed to amplification in the case of liver grafts) after 10 d of culture.

Discussion

In a previous search for myeloid progenitors in normal mouse liver using the same methods subsequently adopted in this study, cells were propagated with GM-CSF that exhibited many characteristics of DC (19). These included the expression of murine DC-restricted cell surface immunophenotypic markers (27). The cells did not, however, conform to the stereotypic features classically associated with ma-

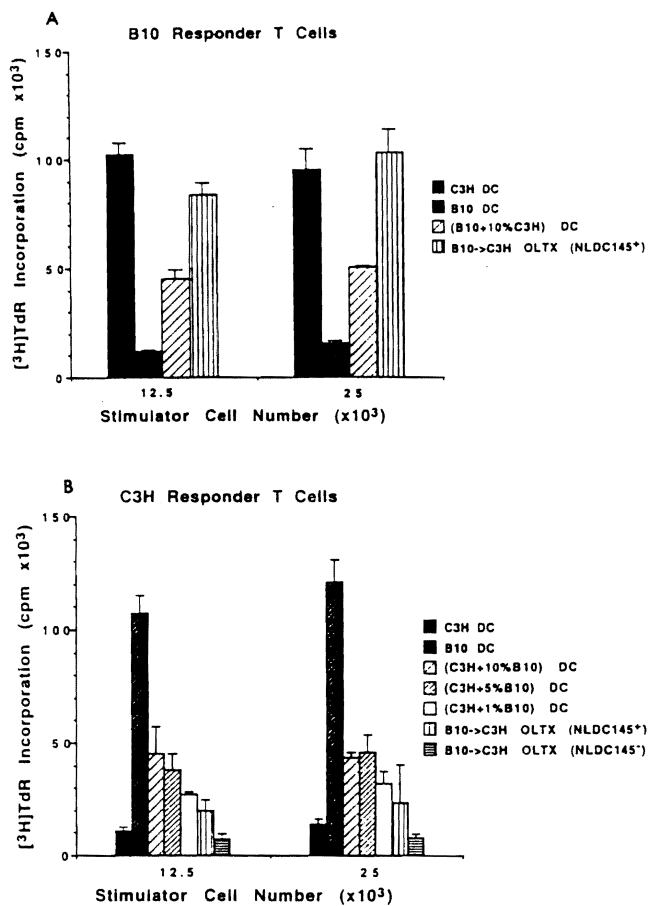


Figure 5. Allostimulatory activity of γ -irradiated, GM-CSF plus IL-4-stimulated, bone marrow-derived DC determined using naive B10 (A) or C3H (B) splenic T cells as responders. The cells were harvested from 10-d cultures and set up with 2×10^5 responder T cells. Syngeneic or allogeneic cells, mixtures of syngeneic and allogeneic cells, or cells propagated from the bone marrow of OLTx recipients (14 d after transplant) were tested as stimulators. Cultures were maintained for 72 h; $[^3\text{H}]\text{TdR}$ was added 18 h before harvesting. The results are expressed as mean cpm \pm 1 SD and are representative of three separate experiments. 50×10^3 fresh syngeneic spleen cells (C3H or B10) failed to induce a MLR.

ture murine DC (11, 15, 25). Thus, they were MHC Class II^{-dim} in contrast to MHC class II^{bright} spleen-derived DC propagated under similar conditions, and they were poor stimulators of allogeneic T cells. They thus resembled immunologically immature DC (15, 28). When, however, these cells were exposed to the liver microenvironmental extracellular matrix protein type I collagen for 3–4 d in the continued presence of GM-CSF, they assumed more mature DC characteristics. These included strong cell surface MHC class II expression, loss of phagocytic ability, and acquisition of potent allostimulatory activity (19). In addition, we observed that 1 to at least 60 d after these immature DC progenitors were injected locally or systemically into MHC and non-MHC disparate recipients, donor cells that were MHC class II^{bright} could be detected readily within T cell areas of the host's lymphoid tissues (20).

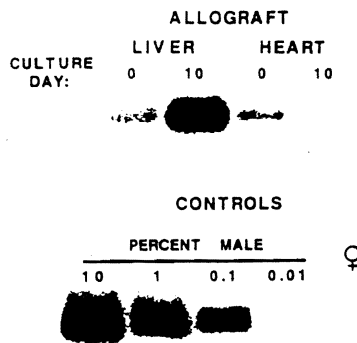


Figure 6. Detection of the donor Y chromosome in freshly isolated (day 0) and 10-d cultured bone marrow cells from female (C3H) recipients of male (B10) livers or hearts. The animals were killed 14 d or 8 d, respectively, after transplantation. In this strain combination, liver allografts are accepted spontaneously, whereas heart grafts are rejected with a median survival time of 8 d. Growth of donor-derived cells is again evident in the DC cultures propagated from the bone marrow of liver allograft recipients, whereas there is little evidence of survival of male cells in the cultures from heart graft recipients. The results are representative of three separate experiments. The technical details are as described in Materials and Methods.

These observations suggested, amongst other implications, a mechanism for liver DC precursor expansion and a possible basis for the establishment and perpetuation of donor-derived cell microchimerism after OLTx. The findings have now been extended to the context of whole organ transplantation. In the present study, an MHC class I- and II-disparate mouse strain combination (B10 \rightarrow C3H) that accepts liver grafts spontaneously was used to determine whether liver-derived DC progenitors could be propagated from recipient lymphoid tissue. To confirm the presence of donor-derived cells, we first examined with flow cytometry the frequency of donor and recipient MHC class I⁺ leukocytes in freshly isolated cell suspensions from the bone marrow, spleen, thymus, and liver 14 d after OLTx. As anticipated, donor class I⁺ cells were rare ($\leq 1\%$) in the freshly isolated cell populations isolated from recipient primary and secondary lymphoid tissues. A higher incidence of donor class I⁺ NPC (5–9%) was detected in the liver allograft itself. When, however, cells were harvested from recipient bone marrow 14 d after OLTx and then cultured for 10 d in GM-CSF, a significant proportion of the developing DC exhibited donor phenotype. The same phenomenon was observed with cells propagated from the spleen, although the apparent growth of donor-derived DC was less striking. The expression of MHC class II on these cells could most accurately be studied after immunochemical staining of cytospin preparations. Similar to the class I results, donor class II⁺ cells were readily detected in the GM-CSF-stimulated cultures from bone marrow and spleen, both of which are hemopoietic tissues. In contrast, although donor class I⁺ and II⁺ cells were also detected in freshly isolated thymocyte preparations, it proved consistently difficult to demonstrate *ex vivo* growth of DC progenitors from the thymuses of liver-allografted mice.

Definitive evidence for the growth of donor liver-derived

cells from the bone marrow (especially) and spleen was obtained by detection of the presence of the (donor) Y chromosome by PCR amplification and Southern blotting. These revealed a level of chimerism in the GM-CSF-stimulated cultures of ~1-10% (Fig. 3). Definitive evidence that donor-positive cells were DC was obtained by demonstration of the Y chromosome (Fig. 4) and allostimulatory activity for recipient strain naive T cells (Fig. 5 B) in highly purified NLDC 145⁺ cell populations. In marked contrast to these findings, donor-derived cells could not be propagated from the bone marrow, spleen, or thymus of unmodified cardiac allograft recipients rejecting their transplants at 8 d. Although donor-derived cells were detected in the freshly isolated bone marrow, these cells clearly did not comprise sufficient numbers of GM-CSF-responsive cells to allow perpetuation of donor signal for 10 d in culture. An implication of these observations is that acceptance of an organ allograft may be dependent on sustained chimerism comprising sufficient numbers of the appropriate donor-derived progenitor cell population(s).

Our observations appear to explain the persistence of low-level chimerism that does not require host immunosuppression in the B10 → C3H liver transplant model. They are also consistent with the adult liver containing comparatively large numbers of (potential migratory) hemopoietic cells (29), including DC progenitors with the capacity to propagate *ex vivo* in response to appropriate growth factors. The observation that organ transplantation permits the growth of donor as well as recipient progenitor DC is congruent with the paradigm of bidirectional immune reactivity, which, we have suggested, may explain whole-organ graft acceptance and acquired tolerance in a different way than previously envisioned (1, 5, 6, 8, 30). We postulate that the peripheral tissues and the graft itself become repositories of precursor cells of myeloid and probably other lineages, or perhaps even of pluripotent stem cells. The presence of these precursor cells can potentially be identified, as in this study, by their patterns of differentiation under controlled culture conditions.

The functional role of the precursors of chimeric DC is currently being investigated. Others have demonstrated a veto

function for either subpopulations of murine DC (31) or putative immature DC in primate allogeneic bone marrow (32, 33). Moreover, there is strong evidence that the reduction of MHC class I-specific cytotoxic responses in mice injected with allogeneic donor lymphoid cells is a function of donor-deletional APC that inactivate class I-reactive CTL precursors that recognize them (34). The presence of donor-derived DC for up to 1 yr in rodents tolerant to their allografts (5, 6) and for many years after OLTx in humans (2) has been demonstrated. Moreover, using techniques described in the present study, we have preliminary evidence that donor-derived cells can be propagated from the bone marrow of unmodified murine liver allograft recipients up to 5 mo after transplant (our unpublished observations). The magnitude, tissue-specific site dependency, replicative capacity, and maturational stage of chimeric DC (including cell surface expression of key T cell-costimulatory molecules [35, 36]) that may be necessary to mediate postulated tolerizing effects of these cells have not been established.

Our failure to propagate chimeric DC progenitors from the thymus is of particular interest. Previous reports have shown that either intrathymic (37) or intravenous (38) donor bone marrow cell infusion in antilymphocyte serum/anti-thymocyte globulin-treated mice results in only a comparatively low level of thymic chimerism that can no longer be detected after 3 wk. Indeed, permanent cardiac graft survival in mice is maintained despite the apparent absence of donor bone marrow cell-derived chimerism in the thymus (37). Moreover, the thymus is not required for the induction of liver-induced transplantation tolerance (39). Thus, chimerism in the periphery may be more important in maintaining a tolerant state (40-42). Conceivably, the recipient bone marrow may provide a repository of donor-derived DC progenitors with the potential to migrate to key peripheral sites. The unique observations in this study provide an opportunity for elucidation of the role of donor-derived cells in the induction and maintenance of donor-specific unresponsiveness after organ transplantation.

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References

- Starzl, T.E., A.J. Demetris, N. Murase, S. Ildstad, C. Ricordi, and M. Trucco. 1992. Cell migration, chimerism, and graft acceptance. *Lancet*. 339:1579-1582.
- Starzl, T.E., A.J. Demetris, M. Trucco, A. Zeevi, H. Ramos, P. Terasaki, W.A. Rudert, M. Kocova, C. Ricordi, S. Ildstad, and N. Murase. 1993. Chimerism and donor specific nonreactivity 2 to 27 years after kidney allotransplantation. *Transplantation (Baltimore)*. 55:1272-1277.
- Schlitt, H.J., J. Hundrieser, M. Hisanaga, K. Uthoff, M. Karck, T. Wahlers, K. Wonigeit, and R. Pichlmayr. 1994. Patterns of donor-type microchimerism after heart transplantation. *Lancet*. 343:1469-1471.
- Suberielle, C., S. Caillat-Zucman, C. Legendre, C. Bodemer, L.-H. Noel, H. Kreis, and J.-F. Bach. 1994. Peripheral microchimerism in long-term cadaveric-kidney allograft recipients. *Lancet*. 343:1468-1469.
- Demetris, A.J., N. Murase, S. Fujisaki, J.J. Fung, A.S. Rao, and T.E. Starzl. 1993. 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.* 25:3337-3344.
- Qian, S., A.J. Demetris, N. Murase, A.S. Rao, J.J. Fung, and T.E. Starzl. 1994. Murine liver allograft transplantation: tolerance and donor cell chimerism. *Hepatology*. 19:916-924.
- Demetris, A.J., N. Murase, and T.E. Starzl. 1992. Donor dendritic cells after liver and heart allotransplantation under short-term immunosuppression. *Lancet*. 339:1610.
- Starzl, T.E., A.J. Demetris, M. Trucco, N. Murase, C. Ricordi, S. Ildstad, H. Ramos, S. Todo, A. Tzakis, J.J. Fung, et al. 1993. Cell migration and chimerism after whole organ transplantation: the basis of graft acceptance. *Hepatology*. 17:1127-1152.
- Austyn, J.M., M.W. Kupiec-Weglinski, D.F. Hankins, and P.J. Morris. 1988. Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone. *J. Exp. Med.* 167:646-651.
- Larsen, C.P., R.M. Steinman, M. Witmer-Pack, D.F. Hankins, P.J. Morris, and J.M. Austyn. 1990. Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* 172:1483-1493.
- Steinman, R.M., and Z.A. Cohn. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J. Exp. Med.* 139:380-397.
- Austyn, J.M., and C.P. Larsen. 1990. Migration patterns of dendritic leukocytes. Implications for transplantation. *Transplantation (Baltimore)*. 49:1-7.
- Steinman, R.M., D.S. Lustig, and Z.A. Cohn. 1974. Identification of a novel cell in peripheral lymphoid organs of mice. III. Functional properties in vivo. *J. Exp. Med.* 139:1431-1445.
- Fossum, S. 1989. The life history of dendritic leukocytes (DL). In *Current Topics in Pathology*. O.H. Ivesson, editor. Springer-Verlag, Berlin. 101-124.
- Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271-296.
- Inaba, K., R.M. Steinman, M.W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* 175:1157-1167.
- Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693-1702.
- Lu, L., M. Hsieh, T.B. Oriss, P.A. Morel, T.E. Starzl, A.S. Rao, and A.W. Thomson. 1995. Generation of dendritic cells from mouse spleen cell cultures in response to GM-CSF: immunophenotypic and functional analyses. *Immunology*. 84:127-134.
- Lu, L., J. Woo, A.S. Rao, Y. Li, S.C. Watkins, S. Qian, T.E. Starzl, A.J. Demetris, and A.W. Thomson. 1994. Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturational development in the presence of type-1 collagen. *J. Exp. Med.* 179:1823-1834.
- Thomson, A.W., L. Lu, V.M. Subbotin, Y. Li, S. Qian, A.S. Rao, J.J. Fung, and T.E. Starzl. 1995. In vitro propagation and homing of liver-derived dendritic cell progenitors to lymphoid tissues of allogeneic recipients. *Transplantation (Baltimore)*. 59:544-551.
- Qian, S., J.J. Fung, A.J. Demetris, S.T. Ildstad, and T.E. Starzl. 1991. Orthotopic liver transplantation in the mouse. *Transplantation (Baltimore)*. 52:562-564.
- Ono, K., and E.S. Lindsey. 1968. Improved technique of heart transplantation in rats. *J. Thorac. Cardiovasc. Surg.* 57:225-229.
- Sallusto, F., and A. Lanzavecchia. 1994. 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.* 179:1109-1118.
- Starzl, T.E., A.J. Demetris, M. Trucco, H. Ramos, A. Zeevi, W.A. Rudert, M. Kocova, C. Ricordi, S. Ildstad, and N. Murase. 1992. Systemic chimerism in human female recipients of male livers. *Lancet*. 340:876-877.
- Gubbay, J., J. Collignon, P. Koopman, B. Capel, A. Economou, A. Münsterberg, N. Vivian, P. Goodfellow, and R. Lovell-Bodge. 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature (Lond.)*. 346:245-250.
- Steinman, R.M., and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* 137:1142-1162.
- Agger, R., M.T. Crowley, and M.D. Witmer-Pack. 1990. The surface of dendritic cells in the mouse as studied with monoclonal antibodies. *Int. Rev. Immunol.* 6:89-101.
- Austyn, J.M. 1993. The initiation of immune responses and allograft rejection. In *Immunology of Heart and Lung Transplantation*. M.L. Rose and M.H. Yacoub, editors. Little, Brown & Co., Boston. 22-41.
- Sakamoto, T., T. Saizawa, A. Mabuchi, Y. Norose, T. Shoji, and K. Yokomuro. 1992. The liver as a potential hematolymphoid organ examined from modifications occurring in the systemic and intrahepatic hematolymphoid system during liver regeneration after partial hepatectomy. *Regional Immunology*. 4:1-11.
- Starzl, T.E., A.J. Demetris, N. Murase, A.W. Thomson, M. Trucco, and C. Ricordi. 1993. Donor cell chimerism permitted by immunosuppressive drugs: a new view of organ transplantation. *Immunol. Today*. 14:326-332.
- Vremec, D., M. Zorbas, R. Scollay, D.J. Saunders, C.F. Ardavin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J. Exp. Med.* 176:47-58.

32. Thomas, J.M., F.M. Carver, P.R.G. Cunningham, L.C. Olson, and F.T. Thomas. 1991. Kidney allograft tolerance in primates without chronic immunosuppression—the role of veto cells. *Transplantation (Baltimore)*. 51:198–207.
33. Thomas, J.M., F.M. Carver, J. Kasten-Jolly, C.E. Haisch, L.M. Rebellato, U. Gross, S.J. Vore, and F.T. Thomas. 1994. Further studies of veto activity in rhesus monkey bone marrow in relation to allograft tolerance and chimerism. *Transplantation (Baltimore)*. 57:101–115.
34. Kiziroglu, F., and R.G. Miller. 1991. In vivo functional clonal deletion of recipient CD4⁺ T helper precursor cells that can recognize class II MHC on injected donor lymphoid cells. *J. Immunol.* 146:1104–1112.
35. Hart, D.N.J., C. Starling, V.L. Calder, and N.S. Fernando. 1993. B7/BB-1 is leucocyte differentiation antigen on human dendritic cells induced by activation. *Immunology*. 79:616–620.
36. Larsen, C.P., S.C. Ritchie, R. Hendrix, P.S. Linsley, K.S. Hathcock, R.J. Hodes, R.P. Lowry, and T.C. Pearson. 1994. Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. *J. Immunol.* 22:5208–5219.
37. Odorico, J.S., C.F. Barker, A.M. Posselt, and A. Naji. 1992. Induction of donor-specific tolerance to rat cardiac allografts by intrathymic inoculation of bone marrow. *Surgery (St. Louis)*. 112:370–377.
38. Smith, J.P., J. Kasten-Jolly, L.J. Field, and J.M. Thomas. 1994. Assessment of donor bone marrow cell-derived chimerism in transplantation tolerance using transgenic mice. *Transplantation (Baltimore)*. 58:324–329.
39. Kobayashi, E., N. Kamada, L. Delriviere, R. Lord, S. Goto, N.I. Walker, S. Enosawa, and M. Miyata. 1995. Migration of donor cells into the thymus is not essential for induction and maintenance of systemic tolerance after liver transplantation in the rat. *Immunology*. 84:333–336.
40. Zamoyska, R., H. Waldmann, and P. Matzinger. 1989. Peripheral tolerance mechanisms prevent the development of autoreactive T cells in chimeras grafted with two minor incompatible thymuses. *Eur. J. Immunol.* 19:111–117.
41. Kosaka, H., and J. Sprent. 1993. Tolerance of CD8⁺ T cells developing in parent → F₁ chimeras prepared with supralethal irradiation: step-wise induction of tolerance in the intrathymic and extrathymic environments. *J. Exp. Med.* 177:367–378.
42. Maeda, T., M. Eto, Y. Nishimura, K. Nomoto, Y.Y. Kong, and K. Nomoto. 1993. Role of peripheral hemopoietic chimerism in achieving donor-specific tolerance in adult mice. *J. Immunol.* 150:753–762.