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IDENTIFICATION OF DONOR-DERIVED DENDRITIC CELL PROGENITORS IN BONE MARROW OF SPONTANEOUSLY TOLERANT LIVER ALLOGRAFT RECIPIENTS[1,2]

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Multilineage donor-derived hematopoietic cell chimerism is a persistent feature of spontaneously tolerant mouse liver allograft recipients. We have shown previously that normal liver-derived precursors of "chimeric" dendritic cells (DC) propagated in vitro migrate in vivo to T-dependent areas of allogeneic lymphoid tissue, where they or their progeny appear to persist indefinitely. In this study, granulocyte-macrophage colony-stimulating factor (GM-CSF)+interleukin-4 (IL-4) were used to propagate DC progenitors from freshly isolated mouse bone marrow. The progenitor cells gave rise in 7–10 days to potent antigen-presenting cells (APC) that stimulated naive allogeneic T cells in primary mixed leukocyte cultures (MLC). The culture method, together with the reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of donor and recipient strain major histocompatibility complex (MHC) class II mRNA was used to test whether donor-derived DC could be propagated from the bone marrow of unmodified, orthotopic liver allograft recipients. Freshly isolated bone marrow from these transplanted animals contained small numbers of donor cells and responded to GM-CSF+IL-4 stimulation. In addition to cells expressing recipient (B10) phenotype (H-2K^{b+}; Ia^{b+}), a minor population of donor (B10.BR)-derived cells (H-2K^{k+}; Ia^{k}) were also propagated from liver graft recipients euthanized two weeks posttransplant. DC sorted from these cultures exhibited stimulatory activity for recipient strain T cells consistent with a low level (<1%) of donor DC propagation. The immunologic role of donor-derived DC progenitors in liver allograft recipients and its relation to the induction and maintenance of donor-specific unresponsiveness remains to be determined.

It has been postulated that the reduced immunogenicity of orthotopic liver allografts compared with those of other solid organs 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 DC* (*1*). In animals and in man, interstitial DC, such as those seen in normal liver, are believed to arise from proliferating bone marrow–derived precursors (*2*), that can be induced to proliferate in vitro in response to GM-CSF (*3*). In this study, we present cellular and molecular evidence that mouse liver allografts export DC precursors that can be detected in the bone marrow of nonimmunosuppressed recipients. These cells can be induced to proliferate and mature into potent APC in response to GM-CSF+IL-4. This observation is congruent with the possibility that donor hematopoietic cell chimerism may play a key role in liver allograft acceptance and acquired transplantation tolerance.

MATERIALS AND METHODS

Animals. Male B10.BR (H-2^{k}, I-E^{+}) and C57BL/10 SnJ (B10, H-2^{b}, I-E^{-}) mice 10–12 weeks old were purchased from The Jackson Laboratory, Bar Harbor, ME. Orthotopic liver transplantation (OLTx) was performed from B10.BR donors to B10 recipients as described previously (*4*), with minor modifications. No immunosuppressive therapy was used. The animals (three per group in each experiment) were euthanized 14 days after OLTx. Before harvesting of tissue from transplanted animals, whole body perfusion was performed (*5*) to minimize blood contamination. Pooled bone marrow cell suspensions were prepared in RPMI-1640 (Gibco, Grand Island, NY) using conventional methods.

Culture of bone marrow-derived cells with GM-CSF+IL-4. Cells (2–5×10^{6}) in 2 ml RPMI-1640 complete medium supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS; Gibco), GM-CSF (4 ng/ml) and IL-4 (1000 U/ml) (each cytokine from Schering-Plough, Kenilworth, NJ) were cultured per well in 24-well plates. The procedure for propagation of DC from mouse bone marrow was similar to that described by Inaba et al. (*3*) with minor modifications (*5*). IL-4 was added to promote growth of DC and to maximize the development of potent APC function (*6*). The cytokine-containing medium was refreshed every two days. Morphological, phenotypic and functional analyses of typical, single, non-adherent mononuclear cells were performed after 8–10 days.

Staining of cell surface antigens. Cell surface antigens were analyzed by immunofluorescence staining and cytofluorography on a FACScan (Becton Dickinson & Co., Mountain View, CA) as described

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* Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin-4; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLC, mixed leukocyte culture; PCR, polymerase chain reaction; RT, reverse transcriptase; OLTx, orthotopic liver transplantation.

(*5*, *7*). Donor and recipient MHC class I and class II positive cells were identified using biotin-conjugated mouse anti-mouse monoclonal antibodies (mAbs) (PharMingen, San Diego, CA) with FITC streptavidin (Jackson Immunoresearch Lab. Inc., West Grove, PA) as the secondary reagent. Appropriate rat, hamster or mouse immunoglobulin isotype controls were performed in each experiment. Background staining was uniform between samples.

Detection of donor and recipient MHC class II mRNA in cell cultures by RT-PCR analysis. Total RNA was prepared from either freshly isolated or cultured bone marrow cells by standard methods using RNAzol B (Biotecx, Houston, TX) (*8*). RNA concentrations were determined by measuring the absorbance at 260 nm. cDNA was synthesized by using 3 μg of total RNA template with Moloney murine leukemia virus reverse transcriptase (RT) (Life Technologies) and Oligo dT primer in a final volume of 10 μl. Using 300 ng of this first-strand cDNA as template, PCR amplification was conducted for 30 cycles (94°C×1 min, 59°C×1 min, 72°C×1 min) with Taq DNA polymerase and a model 480 Thermal Cycler (both from Perkin-Elmer, Norwalk, CT) using oligonucleotide amplimers targeted to the coding region sequences of cDNA for Ia^b and Ia^k (*9*, *10*). These primers were 20 nucleotides long with the following sequences:

Ia^b forward-5′AGTTTGGCCAATTGGCAAGC3′
Ia^k forward-5′AGTTTGCTCAACTGAGAAGA3′
Ia reverse-5′CCACCTTGCAGTCATAAATG3′

The predicted PCR products span from 198 to 554 bp for Ia^b and from 244 to 600 bp for Ia^k to yield the same predicted PCR product size of 356 bp. Ethidium bromide–stained 2% agarose gels were used to analyze the PCR products. RT-PCR for β-actin served as a control for cDNA synthesis.

Sorting of dendritic cells. Before staining with mAb, the cultured bone marrow–derived cells were incubated with 10% v/v normal goat serum (Vector, Burlingame, CA) in Hanks balanced salts solution (HBSS) (Gibco) at 4°C for 30 min to eliminate nonspecific binding. The cells were then washed once in HBSS containing 0.1% v/v bovine serum albumin (Sigma) and resuspended at 40×10^6/ml in the same buffer. A saturating concentration of the mouse DC restricted mAb NLDC-145 (a gift from Dr. R. M. Steinman, The Rockefeller University, New York, NY) or rat IgG2a isotype control (Sigma) was added and the cells incubated for 45 min at 4°C. After two washes in HBSS, the cells were stained with FITC-conjugated goat anti-rat IgG (Sigma) under the same conditions. They were then washed and resuspended in complete RPMI-1640 media supplemented with 2% v/v FBS for 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 FACStarPlus cell sorter (Becton Dickinson). Purities of both populations >90% were attained.

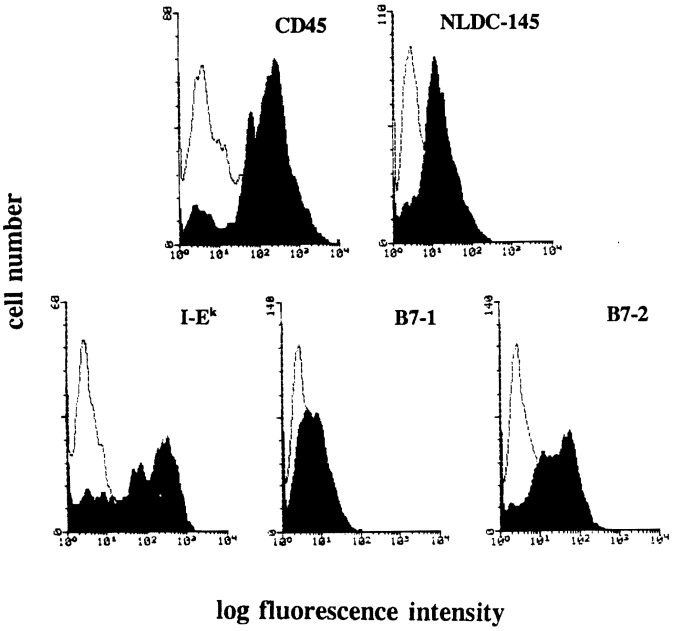

FIGURE 1. The expression of selected cell surface immunophenotypic markers on GM-CSF+IL-4–stimulated mouse (B10.BR) bone marrow–derived DC 10 days after the initiation of cultures. Unshaded profiles denote appropriate immunoglobulin isotype controls. The results are representative of 3 separate experiments.

Mixed leukocyte cultures (MLC). Three day, one-way MLC (2×10^5 responder T cells per well in 96-well, round-bottomed microculture plates) were performed as described (*5*). Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS in 5% CO_2 in air. Various numbers of γ-irradiated (20 Gy) stimulator cells propagated with GM-CSF+IL-4 from donor (B10.BR) or recipient (B10) strain animals or from the bone marrow of liver allograft recipients were added. To assist in 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. Normal B10 or B10.BR splenic T cells served as responders. [^{3}H]TdR (1 μCi) was added to each well, 18 hr before harvesting onto glass fiber disks using a multiple cell harvester. [^{3}H]TdR uptake was determined using a liquid scintillation counter. Each measurement was performed in triplicate and data presented as mean counts per minute (cpm)±1SD.

RESULTS

Immunophenotypic analysis of bone marrow–derived DC. Flow cytometric analysis of 10-day cultured bone marrow–derived cells was performed after either direct or indirect immunofluorescence staining using an extensive panel of mAbs as described (*5*). Staining for cells of lymphoid lineage (CD3$^+$, CD4$^+$, CD8$^+$, B220$^+$, and NK1.1$^+$) was absent. The cells expressed CD45 (leukocyte-common antigen), MHC class I, heat-stable antigen (J11D), CD54 (intercellular adhesion molecule-1), CD11b (MAC-1), CD44 (nonpolymeric determinant of Pgp.1 glycoprotein), and the mouse DC-restricted markers NLDC-145 (interdigitating cells), 33D1 and CD11c (N418; β2-integrin). The macrophage antigen F4/80 and FcγRII (CD32) were also expressed as described previously for GM-CSF-stimulated mouse bone marrow–derived DC (*3*). In addition, the cells exhibited high levels of MHC class II, and low and moderate levels, respectively, of the costimulatory molecules B7–1 and B7–2. A restricted immunophenotypic profile of these cells depicting DC characteristics is shown in Figure 1.

Detection of cells expressing donor phenotype in freshly isolated and GM-CSF+IL-4–stimulated bone marrow from liver allograft recipients. To identify cells expressing donor phenotype in freshly isolated bone marrow from nonimmunosuppressed B10 mice 14 days after OLTx from B10.BR donors, mAbs directed against donor (H-2K^k) and recipient (H-2K^b) MHC class I antigens were used. In addition, primers specific for donor or recipient MHC class II were used to detect mRNA transcripts by RT-PCR. In allografted mice, the incidence of freshly isolated bone marrow cells expressing donor MHC class I determined by flow cytometry was very low (approx. 1%). That of the recipient was, as expected, consistently very high (>84%). RT-PCR analysis of MHC class II mRNA expression in freshly-isolated bone marrow also revealed the presence of donor cells (Fig. 2).

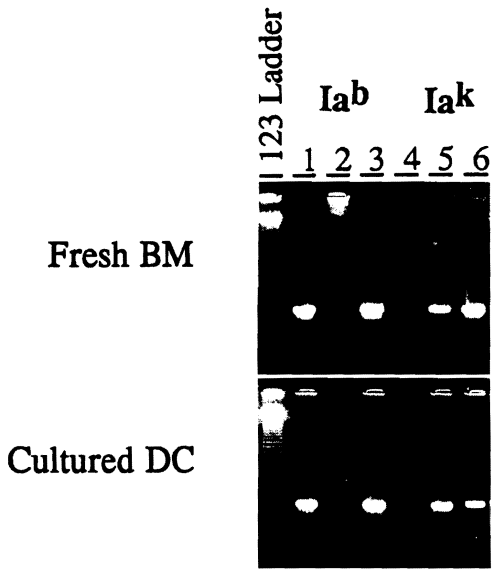

FIGURE 2. Demonstration of mRNA for donor (Ia^k) and recipient strain (Ia^b) MHC class II by RT-PCR analysis. RNA was isolated either from fresh bone marrow (BM) (upper panel) or from 10-day bone marrow–derived DC propagated in GM-CSF+IL-4 as described in *Materials and Methods* (lower panel). (1) Normal B10 (H-2^b); (2) Normal B10.BR (H-2^k); (3) Liver (B10.BR)-allografted B10; (4) Normal B10; (5) Normal B10.BR; (6) Liver (B10.BR)-allografted B10.

We next determined whether DC expressing donor phenotype could be propagated from postulated small numbers of donor-derived progenitor cells in the freshly isolated bone marrow of unmodified liver allograft recipients. In several repeat experiments, both cells expressing recipient MHC class I (H-$2K^{b+}$) and a minor population expressing donor MHC class I (H-$2K^{k+}$) could be identified by cytofluorimetry in the 10-day DC cultures. The cell population was in the correct area for DC as identified by labeling with a specific marker (NLDC-145) and forward and side scatter profiles. Detection of mRNA for donor MHC class II (Ia^k) confirmed the presence of donor-derived cells in the 10-day DC cultures (Fig. 2) propagated from the bone marrow of the transplant recipients.

Allostimulatory activity of donor-derived DC. NLDC-145^+ cells were sorted to at least 90% purity by morphologic and FACScan analysis, and then investigated for their capacity to induce a primary MLR. As shown in Table 1, the purified NLDC-145^+ population propagated from B10 recipients of B10.BR allografts strongly stimulated B10.BR responders, but also stimulated a much more modest response in recipient strain B10 T cells (Table 2). The extent of stimulation ($P<0.01$ compared with negatively sorted cells or syngeneic DC) was similar to that achieved with "artificial mixtures" of GM-CSF+IL-4 stimulated cells containing 1% donor strain and 99% recipient strain DC (Table 2).

DISCUSSION

We have previously demonstrated myeloid progenitors in normal mouse liver, using similar methods to those used in the present study (*5*). Thus, DC progenitors were propagated from normal liver nonparenchymal cells in response to GM-CSF (*5*). Following their local or systemic injection into unmodified MHC and non-MHC disparate recipients, these cells homed to T cell areas of the host's lymphoid tissues (*11*), where at least some could be detected by cell surface expression of donor MHC class II. These observations suggested a possible basis for the establishment and perpetuation of donor-derived cell (DC) microchimerism after OLTx. In the present study, an MHC class I and II–disparate mouse strain combination (B10.BR→B10) that accepts liver grafts spontaneously was used to determine whether liver-derived DC progenitors could be propagated from recipient lymphoid tissue.

The presence of donor and recipient MHC class I^+ leukocytes, and of cells expressing message for donor or recipient MHC class II was investigated in freshly isolated bone marrow cell suspensions 14 days after OLTx. As anticipated, donor class I^+ cells were rare (about 1%) in the freshly isolated cell populations. When cells were harvested from recipient bone marrow 14 days after OLTx, then cultured for 10 days in GM-CSF+IL-4, a significant proportion of the

TABLE 1. Allostimulatory activity of sorted GM-CSF+IL-4 stimulated bone marrow-derived DC for donor strain (B10.BR) T cells[a]

Stimulators ($\times 10^3$)	S:R[b] cell ratio	cpm±1SD ($\times 10^3$)	Stimulation index
Recipient strain: B10 DC			
12.5	1:16	102.04±5.62	8.6
25.0	1:8	95.22±9.69	9.8
Donor strain: B10.BR DC			
12.5	–	11.81±1.07	1.0
25.0	–	15.63±1.27	1.0
Artificial mixture: B10.BR+10% B10 DC			
12.5	1:160	45.04±4.12	3.8
25.0	1:80	50.73±0.54	1.9
Allograft recipient (B10): B10.BR→B10(OLTx) DC			
12.5	Unknown	83.89±5.23	7.1
25.0	Unknown	103.21±10.61	6.6

[a] The stimulator cells were harvested from 10-day cultures and set up in 72-hr MLC with 2×10^5 responder T cells. Syngeneic or allogeneic cells, artificial mixtures of syngeneic and allogeneic cells, or cells propagated from the bone marrow of liver allograft recipients were tested as stimulators. Fresh syngeneic spleen cells (50×10^3) (B10.BR) did not induce an MLR. The results are representative of 3 separate experiments.

[b] Stimulator: responder.

TABLE 2. Allostimulatory activity of sorted GM-CSF+IL-4-stimulated bone marrow-derived DC for recipient strain (B10) T cells[a]

Stimulators ($\times 10^3$)	S:R cell ratio	cpm±1SD ($\times 10^3$)	Stimulation index
Recipient strain (B10) DC:			
12.5	–	10.72±2.15	1.0
25.0	–	14.08±1.93	1.0
Donor strain (B10.BR) DC:			
12.5	1:16	107.47±7.84	10.0
25.0	1:8	121.17±9.54	8.6
Artificial mixture			
B10+10% B10.BR DC			
12.5	1:160	45.47±11.52	4.2
25.0	1:80	43.08±2.73	3.1
B10+5% B10.BR DC			
12.5	1:320	37.78±7.14	3.5
25.0	1:160	45.77±8.09	3.3
B10+1% B10.BR DC			
12.5	1:1600	27.33±0.80	2.5
25.0	1:800	32.05±5.55	2.3
Allograft recipient (B10)			
B10.BR→B10(OLTx) DC			
12.5	Unknown	19.66±5.23	1.8
25.0	Unknown	23.24±1.73	1.7

[a] The stimulator cells were harvested from 10-day cultures and set up in 72-hr MLC with 2×10^5 responder T cells. Syngeneic or allogeneic cells, artificial mixtures of syngeneic and allogeneic cells, or cells propagated from the bone marrow of liver allograft recipients were tested as stimulators. Fresh syngeneic spleen cells 50×10^3 (B10) did not induce an MLR. The results are representative of 3 separate experiments.

developing DC exhibited donor phenotype. Further evidence for donor-derived DC in cell cultures from allograft recipients was obtained by demonstration of allostimulatory activity for naive recipient strain T cells, using highly purified NLDC-145$^+$ cell populations. The extent of stimulation was similar to that achieved with an "artificial mixture" containing 1% donor strain DC. This suggests that a similar proportion of donor cells were present in the cultured bone marrow population from liver allograft recipients. There may however, be strain differences in growth of donor versus host bone marrow progenitors. To account for this, and to obtain a clearer indication of the growth potential of donor cells in the presence of recipient progenitors, it will be necessary in future experiments to propagate mixed populations of donor and host bone marrow cells in different compositions prior to sorting and testing in the MLR. In a separate study, using a different mouse strain combination (B10→C3H; H-2^b→H-2^k), we have demonstrated propagation of male donor-derived DC bearing the Y chromosome from progenitors in the bone marrow of unmodified female liver allograft recipients (*12*).

Our observations appear to explain the persistence of low level hematopoietic cell chimerism in the mouse orthotopic liver transplant model in the absence of host immunosuppression. They are also consistent with the adult liver containing comparatively large numbers of (potential migratory) hemopoietic cells (*13*), including DC progenitors, with the capacity to propagate ex vivo in response to appropriate cytokine 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, as we have suggested, may explain whole organ graft acceptance and acquired transplantation tolerance (*1, 14, 15*). It appears that the recipients' tissues become repositories of precursor cells of myeloid and probably other lineages, or perhaps even of pluripotent stem cells. As in this study, the presence of these precursor cells can potentially be identified by their patterns of differentiation under the influence of appropriate cytokines.

The functional role of the precursors of chimeric DC is currently being investigated. 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 MHC class I–reactive cytotoxic T cell precursors that recognize them (*16*). Others have demonstrated a veto function for subpopulations of mouse DC (*17*) or putative "immature" DC in primate allogeneic bone marrow (*18*). Furthermore, DC within certain tissues such as the anterior chamber of the eye (*19*) or thymus (*20*) appear to have the capacity to induce antigen-specific tolerance in adult animals.

The presence of donor-derived DC for up to 1 year in rodents tolerant to their hepatic allografts (*21*) and for many years after OLTx in humans (*22*) has been demonstrated. The magnitude, tissue-specific site dependency, replicative capacity, or maturational stage of chimeric DC (including cell surface expression of key T cell costimulatory molecules [*23*]) that may be necessary to mediate postulated tolerizing effects of these cells has not been established. However, cultured bone marrow–derived DC that are deficient in cell-surface expression of costimulatory (B7) molecules can induce alloantigen-specific anergy in allogeneic T cells in vitro (*24*). Moreover, these bone marrow–derived cells and DC progenitors propagated from mouse liver can significantly prolong the survival of heart (*25*) and pancreatic islet allografts (*26*), respectively. Conceivably, following liver transplantation, the recipient bone marrow may provide a repository of donor-derived DC progenitors with the potential to migrate to central or peripheral lymphoid tissue sites and therein to affect the responsiveness of host alloantigen-specific T cells. The observations in this study provide an oppor-

tunity for elucidation of the role of these donor-derived cells in the induction and maintenance of donor-specific unresponsiveness following organ transplantation.

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