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

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Summary

Within 1 wk of liquid culture in granulocyte/macrophage colony-stimulating factor (GM-CSF), normal B10 BR (H-2^k I-E^+) mouse liver nonparenchymal cells (NPC) formed loosely adherent myeloid cell clusters that have been shown to contain dendritic cell (DC) progenitors in similar studies of mouse blood or bone marrow. Mononuclear cell progeny released from these clusters at and beyond 4 d exhibited distinct dendritic morphology and were actively phagocytic. After 6–10 d of culture, these cells strongly expressed CD45, CD11b, heat stable antigen, and CD44. However, the intensity of expression of the DC-restricted markers NLDC 145, 33D1, and N418, and the macrophage marker F4/80, intercellular adhesion molecule 1, and FcγRII was low to moderate, whereas the cells were negative for CD3, CD45RA, and NK1.1. Splenocytes prepared in the same way also had a similar range and intensity of expression of these immunophenotypic markers. Unlike the splenic DC, however, most of the GM-CSF–propagated putative liver DC harvested at 6–10 d expressed only a low level of major histocompatibility complex (MHC) class II (I-E^k), and they failed to induce primary allogeneic responses in naive T cells, even when propagated additionally in GM-CSF and tumor necrosis factor α and/or interferon γ–supplemented medium. However, when 7-d cultured GM-CSF–stimulated liver cells were maintained additionally for three or more days on type-1 collagen-coated plates in the continued presence of GM-CSF, they exhibited characteristics of mature DC: MHC class II expression was markedly upregulated, mixed leukocyte reaction stimulatory activity was increased, and phagocytic function was decreased. Similar observations were made when Ia^+ cells were depleted from the GM-CSF–propagated cells before exposure to collagen. Further evidence that the GM-CSF–stimulated class II^{dim} or class II–depleted hepatic NPC were immature DC was obtained by injecting them into allogeneic B10 (H-2^b I-E^-) recipients. They "homed" to T cell–dependent areas of lymph nodes and spleen where they strongly expressed donor MHC class II antigen 1–5 d later. These observations provide insight into the regulation of DC maturation, and are congruent with the possibility that the migration of immature DC from normal liver and perhaps other organ allografts may help explain their inherent tolerogenicity.

The ubiquitous migration of leukocytes of bone marrow origin from transplanted organs into recipient tissues has been proposed as the first step toward organ allograft "acceptance" and the induction of variable degrees of donor-specific nonreactivity, which may or may not require maintenance immunosuppression (1, 2). In this paradigm, the comparative tolerogenicity and ease of acceptance of different organs is thought to hinge on their quantitative and qualitative endowment with these passenger leukocytes, of which the bone marrow–derived dendritic cell (DC)[1] lineage, defined by Steinman and co-workers (3–5), has been postulated to be the most important (1, 2, 6). The liver, with its comparatively heavy content of DC and other leukocyte lineages, is

[1] *Abbreviations used in this paper:* DC, dendritic cell; NPC, nonparenchymal cell; RT, room temperature.

the most tolerogenic whole organ (7–9) and across most mouse strain combinations, can be transplanted successfully without host immunosuppression (10). Although liver interstitial DC, which are localized in the portal triads, have been characterized by immunohistochemical studies in both rodents (11, 12) and humans (13), there are few published data concerning the in vitro properties of liver-derived DC (14) and no reports of attempts to propagate DC lineage cells from normal liver. Consequently, we undertook a study of mouse liver DC, beginning with the technique of Inaba et al. who used GM-CSF to propagate DC progeny from precursors present in mouse blood (15) or bone marrow (16).

We report here on the immunophenotype and in vitro allostimulatory activity of DC progenitors propagated selectively from GM-CSF–stimulated B10.BR mouse liver nonparenchymal cells (NPC). These cells were compared directly with similarly isolated and propagated spleen-derived DC. The results suggest a possible linkage between hepatic tolerogenicity and the presence, within the liver, of DC progenitors (MHC class II^{dim}) that proliferate rapidly and that resist conventional attempts to induce maturation in vitro using GM-CSF alone or in combination with either TNF-α or IFN-γ. These same cells or Ia-depleted GM-CSF–propagated cells, however, could be induced to differentiate after prolonged exposure to GM-CSF in the presence of type-I collagen, an extracellular matrix protein spatially associated with DC within the normal liver (17). After subcutaneous injection into the footpad or intravenous injection into nonimmunosuppressed normal allogeneic recipients, the immature (MHC class II depleted or class II^{dim}) liver-derived cells also exhibited the capacity, as previously described for DC (18–20), to home to T-dependent areas of secondary lymphoid tissue, where they upregulated their MHC class II expression.

Materials and Methods

Animals. Adult 8–12-wk-old male B10.BR ($H\text{-}2^k$, $I\text{-}E^+$) and C57BL/10SnJ (B10, $H\text{-}2^b$, $I\text{-}E^-$) mice 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.

Isolation of NPC from Liver (See Fig. 1). B10.BR mice were anaesthetized with metofen, swabbed with 70% ethanol, and an abdominal mid-line incision performed. The liver was perfused for 3 min in situ via the inferior vena cava, using 30 ml HBSS (GIBCO BRL, Gaithersburg, MD) and a 22-G intravenous catheter (Critikon Inc., Tampa, FL). 2 ml collagenase solution (Sigma Chemical Co., St. Louis, MO; type IV, 1 mg/ml in HBSS) was then injected via the portal vein. The liver was excised immediately, diced into small pieces, and digested in collagenase solution (20 ml/liver) for 30 min at 37°C, with constant stirring. The digested tissue was then filtered through a 0.1-mm sterile nylon mesh. Cells from two to four livers were pooled.

The cell suspension was centrifuged and washed twice in RPMI-1640 (GIBCO BRL) supplemented with 2 mM/ml glutamine, 0.1 mM/ml nonessential amino acids, 1 mM/ml sodium pyruvate, 20 μM 2-ME and antibiotics (100 U/ml penicillin; 100 mg/ml streptomycin; complete medium) at 400 *g* for 5 min. The cells were resuspended in 7 ml sterile, self-generating Percoll solution (Sigma Chemical Co.; 1.130 relative density) and centrifuged at 4°C for 10 min at 39,000 *g* using a SS34 rotor in a superspeed centrifuge (Sorvall RC-5B; DuPont Instruments, Chadds Ford, PA). The top layer of cells, containing intact hepatocytes and hepatocyte fragments, was removed and discarded. The cell suspension between the upper and lower (erythrocyte) layer was then harvested and washed twice (5 min each in RPMI-1640). These cells constituted the liver NPC population. Control spleen cell populations were also prepared using the same protocol.

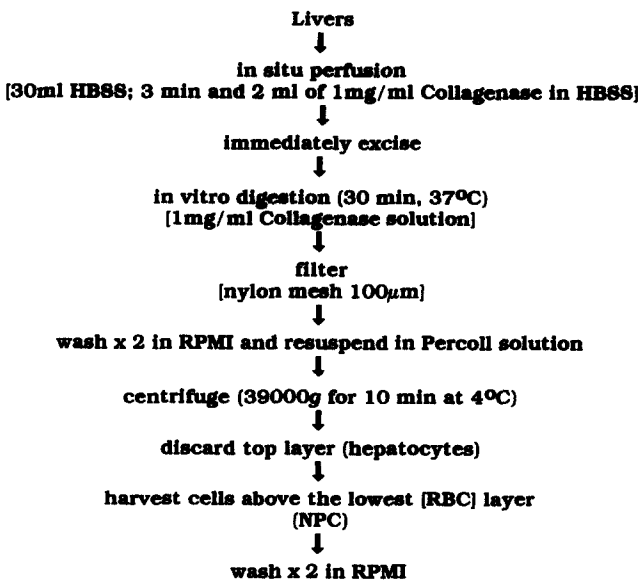

Figure 1. Flow plan for isolation of liver NPC.

Culture of Liver or Spleen-derived Cells with GM-CSF. To test the capacity of liver- or spleen-derived cells to proliferate in response to GM-CSF, 2–5 × 10^5 liver NPC or spleen cells were placed in each well of a 24-well plate in 2 ml of RPMI-1640, supplemented with 10% FCS and 0.4 ng/ml mouse rGM-CSF (R&D Systems, Inc., Minneapolis, MN). The cultures were "fed" every other day by aspirating 50% of the supernatant after gentle swirling, and replenishing with an equivalent volume of fresh GM-CSF–supplemented medium. An objective of these washes was to remove nonadherent granulocytes, without dislodging clusters of developing DC that attached loosely to firmly adherent macrophages. After day 4, granulocytes were no longer significant contaminants of the cultures that were maintained routinely for 7–10 d. In some experiments, in addition to GM-CSF, 500 U/ml recombinant mouse TNF-α (Genzyme Corp., Cambridge, MA) and/or 1,000 U/ml recombinant human IFN-γ (Collaborative Research, Bedford, MA) was also incorporated in the culture medium. Both nonadherent and adherent cells were characterized by Giemsa staining.

Depletion of MHC Class II–positive (Ia^+) Cells. Cells released from growing clusters in 7-d GM-CSF–stimulated cultures of liver-derived cells were depleted of any Ia^+ cells by complement-dependent lysis. The harvested cells were washed three times in HBSS for 5 min each and resuspended at 2 × 10^6/ml in HBSS containing 12.5 μg/ml mouse anti-$I\text{-}E^k$ mAb (PharMingen, San Diego, CA). After incubation at 4°C for 30 min, the cells were washed twice, resuspended in 1:8 low toxicity rabbit complement (Accurate Chemical & Scientific Corp., Westbury, NY), and incubated for a further 30 min at 37°C before two final washes. The efficiency of Ia^+ cell depletion was confirmed by both immunocytochemical staining of cytospins and flow cytometry.

Culture of Putative Liver DC on Collagen-coated Plates. Each well of a 24-well plate was coated with collagen type-1 (50 μg/ml in 0.02 N acetic acid) purified from rat tail tendon (Collaborative Research) for 15 min at 37°C. The collagen solution was then decanted and the wells were allowed to air-dry for 15 min at room temperature (RT) before being washed twice in RPMI-1640 containing 10% fetal bovine serum (FBS). Cells released from growing clusters in 6- or 7-d GM-CSF–stimulated cultures which were free of adherent cells and granulocytes, were transferred to the plates (7×10^5 cells/well) and maintained for a further 3 d in the presence of GM-CSF (0.4 ng/ml). In some experiments, the cells were pretreated to deplete all MHC class II^+ cells (see above).

Flow Cytometric Analysis. Liver or spleen cells (5×10^5/tube) in HBSS with 1% wt/vol BSA and 0.1% sodium azide (both from Sigma Chemical Co.) were stained either by direct or indirect immunofluorescence. Details of the mAbs used are shown in Table 1. Appropriate FITC-conjugated anti-mouse, anti-rat, or anti-hamster secondary Abs were used. Normal hamster serum or the appropriate rat or mouse Ig isotypes were used as negative controls. After staining, cells were fixed in 1% paraformaldehyde in saline before flow cytometric analysis was performed in a FACStar® flow cytometer (Becton Dickinson, San Jose, CA). 5,000 events were acquired for each sample.

Mixed Leukocyte Cultures. To test the immunogenicity of freshly isolated or cultured cells, one-way mixed leukocyte cultures (4×10^5 cells in 200 μl per well in 96-well, round-bottom microculture plates) were performed with γ-irradiated (20 Gy), allogeneic (B10.BR), or syngeneic (B10) liver NPC or spleen cells as stimulators. 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% CO_2 in air; for the final 18 h, 10 μl [^{3}H]TdR (1 μCi) 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 counts per minute ± 1 SD.

Phagocytosis Assay. SRBC (Remel, Lenexa, KS) were washed extensively with ice-cold PBS and opsonized at 37°C for 15 min

Table 1. *Monoclonal Antibody Panel*

Antigen	Species/isotype	Supplier/clone name (American Type Culture Collection [Rockville, MD] No.)
Leukocyte Common Ag		
CD45	Rat IgG2a	M1/9.3.4 (TIB 122)
CD45RA;B220	Rat IgM	RA3-3A 1/6.1 (TIB 146)
MHC		
Class II; I-$E^{k,d,p,r}$	Mouse IgG2a	PharMingen; 14-4-4S (HB32)
DC-restricted		
Lymphoid DC	Rat IgG2b	33D1 (TIB 227)
Interdigitating cell	Rat IgG2a	NLDC-145; Dr. R. M. Steinman, The Rockefeller University, New York
Myeloid (primarily)		
Macrophage	Rat IgG2b	F 4/80 (HB 198); Dr. R. M. Steinman
Lymphoid (primarily)		
Thy 1.2	Rat IgG2a	PharMingen; 53-2.1
CD3-ε	Hamster IgG	PharMingen; 145-2C11
CD4	Rat IgG2a	PharMingen; RM-4-5
CD8α	Rat IgG2a	PharMingen; 53-6.7
Heat stable antigen	Rat IgM	J11D (TIB 183)
NK cells		
NK 1.1	Mouse IgG2a	Dr. W. H. Chambers, University of Pittsburgh
LGL-1	Rat IgG2a	Dr. W. H. Chambers
Receptors/adhesins		
CD32, FcγRII	Rat IgG2b	PharMingen; 2.4G2 (HB 197)
CD11b, MAC-1α unit; C3biR	Rat IgG2b	M1/70. (TIB 128)
CD11c, p150/90	Hamster IgG	N418; Dr. R. M. Steinman
CD44, Pgp-1	Rat IgG2a	PharMingen; 2D2C (TIB 235)
CD54, ICAM-1	Rat IgG2a	Serotec; KAT-1
CD25, p55; IL-2R	Rat IgG1	PC 61 5.3 (TIB 222)

with a 1:100 subagglutinating concentration of rabbit anti-SRBC IgG (Sigma Chemical Co.). The washed SRBC were then suspended in RPMI-1640 and incubated at 37°C for 2 h (8:1) with freshly harvested suspensions of GM-CSF–stimulated, non- and loosely adherent liver-derived cells before and after exposure to collagen. Noningested SRBC were lysed in hypotonic solution (75% RPMI-1640:25% H_2O) for 10 s. The cells were cytocentrifuged, fixed in methanol, and stained with Giemsa. Phagocytic activity (the presence of ≥3 ingested SRBC) was determined "blind" by light microscopic examination. Overnight cultured peritoneal macrophages from the same mouse strain were used as positive controls.

Immunocytochemistry. Cytocentrifuge preparations were stained using avidin-biotin-peroxidase complex (ABC-P; Boehringer Mannheim Corp., Indianapolis, IN) staining procedures. Briefly, specimens were air-dried at RT, before fixing in acetone for 5 min. The slides were then washed in PBS and incubated for 1 h at RT with biotinylated mouse IgG2a anti-mouse I-$E^{k,d,p,r}$ mAb. After three washes of 5 min each in PBS, the slides were incubated with streptavidin-biotin-peroxidase complex for 30 min at RT and the color reaction was developed for 6 min using a peroxidase chromogen kit (AEC; Biomedia Corp., Foster City, CA). Cells were counterstained lightly with hematoxylin. Controls included the omission of Ab and the use of isotype-matched irrelevant mAb.

Dendritic Cell Homing. Ia-depleted or nondepleted cultured B10.BR liver- or spleen-derived cells were washed in RPMI-1640 and injected subcutaneously (1 or 2.5 × 10^5 cells in 50 μl) into one hind footpad or intravenously (10^6 in 200 μl) via the lateral tail vein of normal B10 mice. 1–5 d later, the draining popliteal lymph node and spleen were removed and embedded in Tissue-Tek® (O.C.T. Compound, Miles Inc., Elkhart, IN). 10-μm sections were cut using a cryostat microtome at −30°C and melted directly onto slides at RT. Slides were air-dried at RT overnight, then stored at −70°C until used. Just before staining, the slides were equilibrated at RT, then fixed in acetone before immunoperoxidase staining as described above. Tissue controls included sections of normal recipient strain (B10) tissues.

Results

Isolation of Liver-derived NPC and Their Proliferation in Response to GM-CSF. Approximately 7–10 × 10^6 NPC were isolated per normal mouse liver, with <5% hepatocyte contamination by microscopic examination. A large proportion of the total cell population derived from both the liver and spleen was $B220^+$ (B cells) (~40–50% in liver and 60–80% in spleen) as determined by flow cytometric analysis. These cells accounted largely for the MHC class II^+ ($I\text{-}E^+$) population of comparatively small-sized cells derived from each organ. Within the overall population of NPC, $Thy1.2^+$ T cells were present in smaller numbers compared with B cells (~20% for liver and 30–40% for spleen). It is interesting to note that within the population of small-sized cells, a distinct subpopulation of cells bearing the DC-restricted, interdigitating cell marker NLDC 145 was found in the liver, but not the spleen. Gating for the more granular and larger-sized cells also revealed a much greater population of $NLDC\ 145^+$

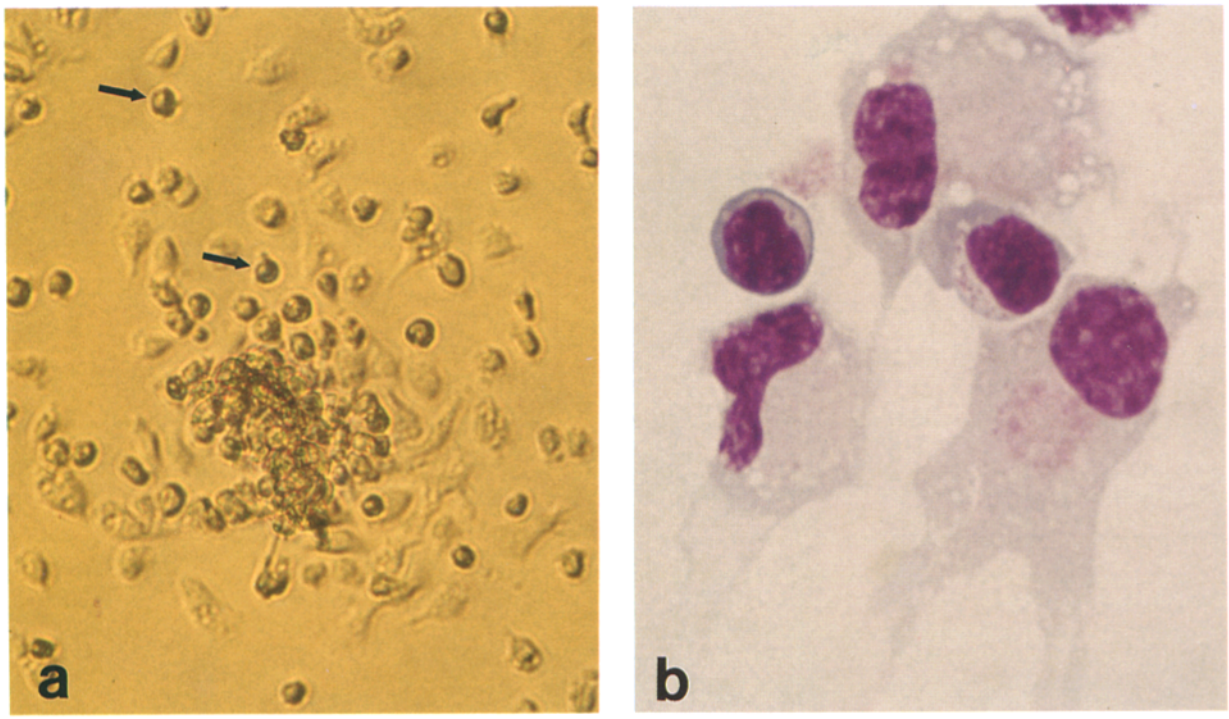

Figure 2. Illustration of the development and isolation of normal mouse liver-derived DC progenitors in liquid cultures supplemented with GM-CSF. (*a*) An early aggregate of proliferating putative DC progenitors (day 4) attached to strongly adherent macrophages and showing typical, loosely adherent cells (*arrows*) that were released from the aggregates. At days 2 and 4 nonadherent granulocytes were removed by gentle aspiration of the culture supernatant and replacement with fresh, GM-CSF–supplemented medium. ×100. (*b*) Giemsa-stained cytocentrifuge preparation showing three released cells (day 6) which exhibit irregular-shaped, eccentric nuclei, variable degrees of vacuolation, absence of prominent cytoplasmic granules, and distinct cytoplasmic processes. ×600.

cells among liver NPC as compared with spleen cells. Indeed, the presence of these NLDC 145$^+$ cells represented the major phenotypic difference between splenocytes and liver NPC using the large panel of mAbs tested (data not shown). After 4-d culture of liver or spleen NPC in GM-CSF, during which time nonadherent granulocytes were removed by gentle washes, growth of cell "clusters" attached to a layer of adherent cells was evident (Fig. 2 *a*). Many dendritic-shaped cells appeared to have been released from the clusters and exhibited sheetlike cytoplasmic processes (Fig. 2 *b*). With more prolonged culture in GM-CSF, these cells detached from the aggregates and many mononuclear cells with a typical dendritic shape were seen either loosely attached or floating in the culture medium. However, in the absence of GM-CSF, no cellular proliferation was seen. Adherent macrophages and fibroblasts also expanded in the liver or spleen cell cultures in the presence of GM-CSF, but remained firmly attached to the plastic surface. The floating or loosely adherent putative DC were harvested by gentle aspiration for further phenotypic or functional analyses. By day 7 of culture, ~2.5 × 10^6 of these cells per mouse liver could be harvested from the cultures.

Microscopic and Immunophenotypic Analysis of GM-CSF–stimulated Liver and Spleen Cells. At the microscopic level, the cells released from proliferating aggregates of GM-CSF–stimulated liver or spleen cells exhibited typical mor-

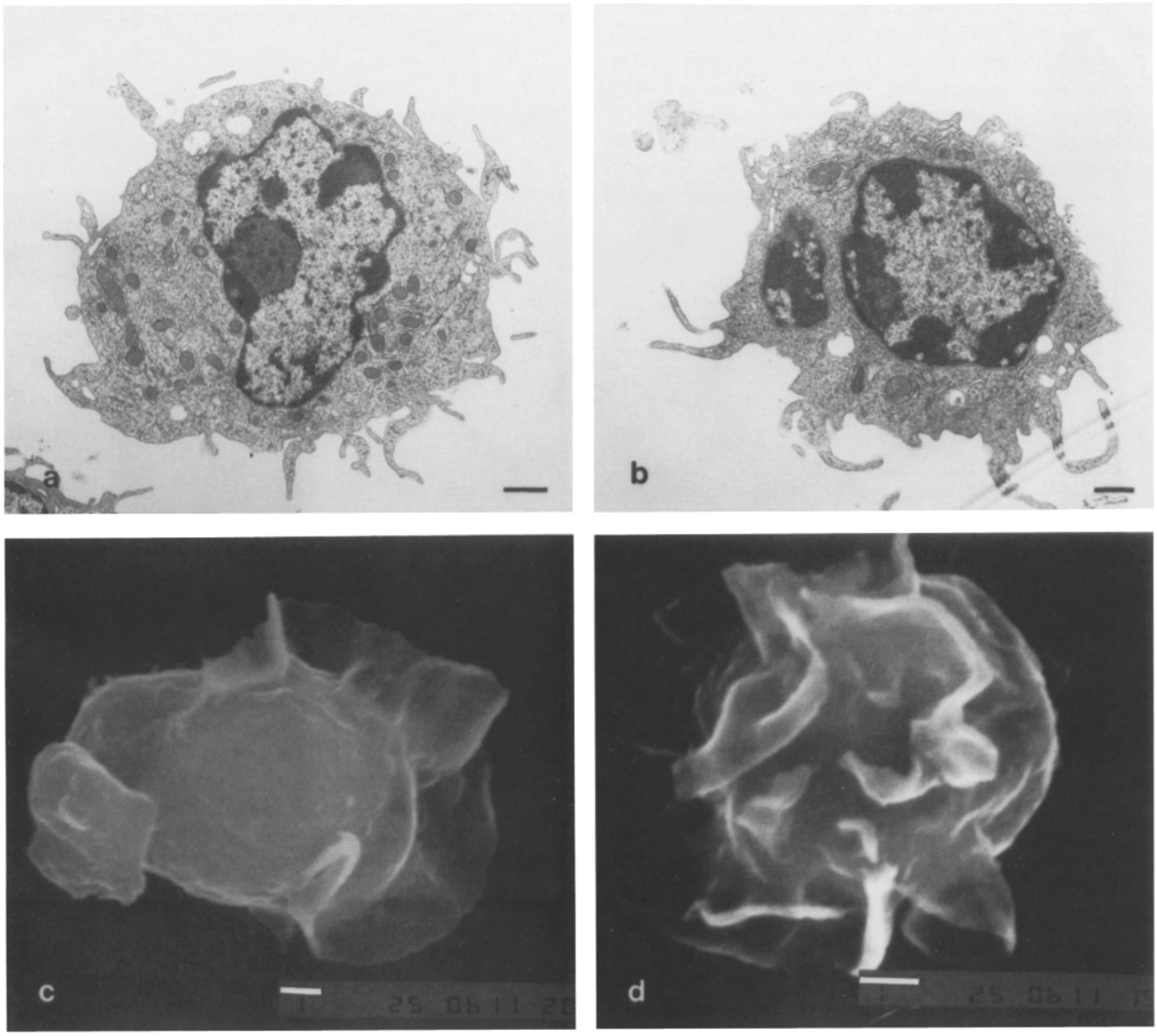

Figure 3. (*a* and *b*) Transmission electron micrographs of loosely adherent cells exhibiting DC morphology released from proliferating aggregates of GM-CSF–stimulated liver-derived cells 9 d after initiation of the cultures. The cells exhibit irregular-shaped nuclei, prominent nucleoli, few electron-dense granules, and numerous mitochondria. (*c* and *d*) Scanning electron micrographs of typical nonadherent cells from the same culture, exhibiting distinct cytoplasmic veils. Bars, 1 μm.

phological features of DC, including (in many but not all cells) irregular-shaped eccentric nuclei, numerous "veil"-like cytoplasmic dendrites, abundant mitochondria, and few electron-dense granules (Fig. 2 *b* and Fig. 3, *a* and *b*). Scanning electron microscopy provided further, definitive evidence of gossamer-like veils (Fig. 3, *c* and *d*). To ascertain the surface phenotype of cells released from proliferating aggregates, flow cytometric analysis was performed after 6–10 d or further periods of culture in GM-CSF. Staining for cells of lymphoid lineage, including NK cells, was absent. As shown in Fig. 4, the floating cells in liver-derived cultures strongly expressed surface antigens that are known to be associated with mouse DC. These included CD45 (leukocyte common antigen), heat stable antigen, intercellular adhesion molecule 1, CD11b (MAC-1), and CD44 (nonpolymeric determinant of Pgp.1 glycoprotein). In addition, staining of weak to moderate intensity was observed for the DC-restricted markers NLDC-145 (interdigitating cells), 33D1, N418, F4/80, and FcγRII. The intensity of expression of these markers on GM-CSF–stimulated spleen cells was similar, except that 33D1 and NLDC 145 were slightly more and less intense, respectively, compared with the liver-derived cells. In contrast to the observation of Inaba et al. (15), however, concerning the

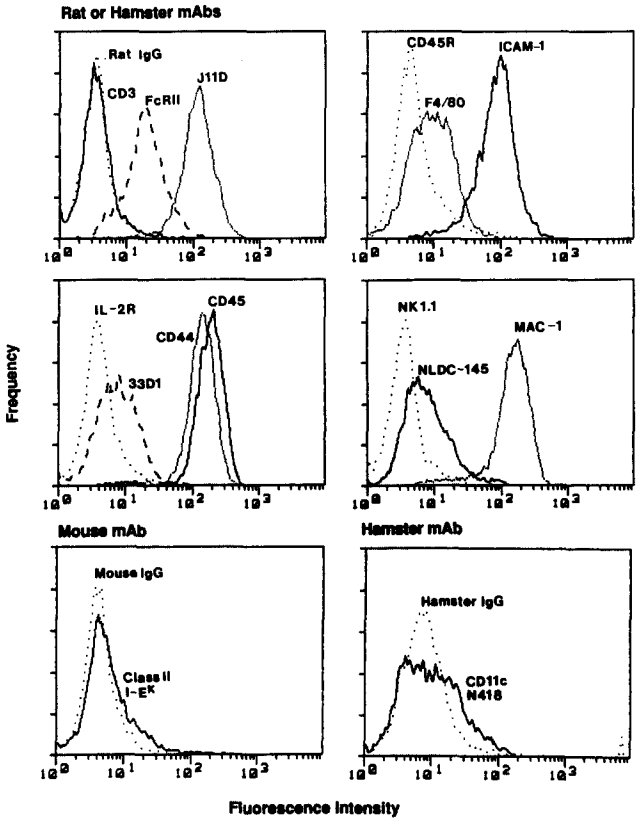

Figure 4. Merged FACScan® immunophenotypic profiles of the GM-CSF–stimulated liver-derived putative DC released from cell aggregates in liquid culture (day 10) and examined using rat, hamster, or mouse mAbs. All data were obtained from the same cell preparation at the same time. The result is representative of four separate experiments performed using cells obtained from 6–10-d cultures. Details of the mAbs are given in Table 1.

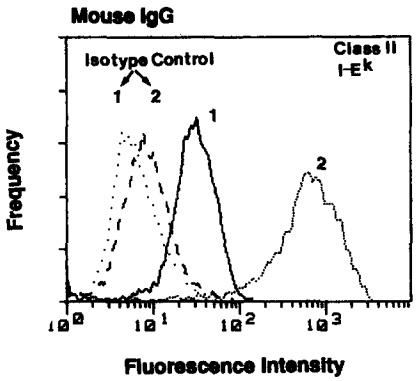

Figure 5. Flow cytometric analysis of MHC class II (I-E^k) expression on (*1*) GM-CSF–stimulated mouse liver putative DC and (*2*) spleen-derived DC released from proliferating aggregates in 10-d cultures. The result is representative of six separate experiments performed using cells obtained from 6–10-d cultures.

progeny of circulating DC precursors, the liver-derived, GM-CSF–stimulated cells expressed only a low level of MHC class II (I-E^k) surface antigen molecule when compared with GM-CSF–stimulated spleen cells propagated under the same conditions (Fig. 5). The intensity of I-E^k expression could not be increased on either the liver- or spleen-derived cells by increasing the concentration of GM-CSF (0.4–0.8 ng/ml) and/or by extending the period of culture for up to 4 wk. The low intensity of I-E^k expression on the liver cell popu-

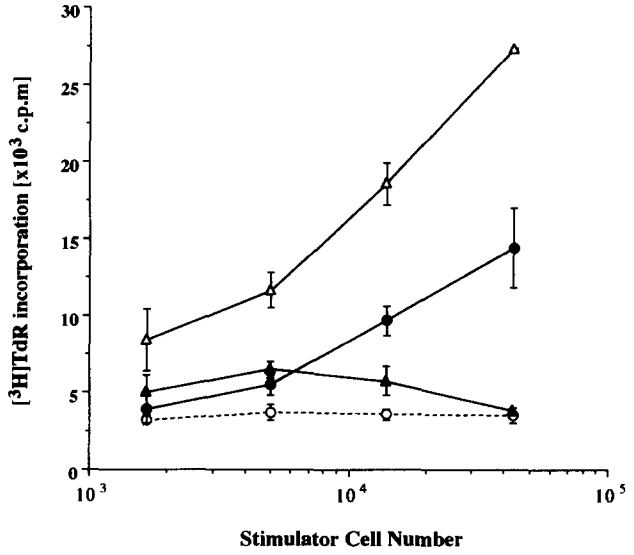

Figure 6. Allostimulatory activity of γ-irradiated, GM-CSF–stimulated B10.BR mouse liver putative DC (▲) or splenic DC (△), using naive B10 (I-E$^-$) splenic T cells as responders. The nonadherent cells were harvested from 10-d GM-CSF–stimulated cultures and set up at various concentrations with 4 × 10^5 responder T cells. Cultures were maintained for 72 h; [^{3}H]TdR was added 18 h before harvesting. The MLR stimulatory activity of freshly isolated allogeneic (B.10 BR [●]) and syngeneic (B10 [○]) spleen cells is also shown. The results are expressed as mean counts per minute (*c.p.m.*) ± 1 SD and are representative of at least three separate experiments.

lation suggested that these proliferating cells, though possessing several surface markers indicative of developing DC, were still at a phenotypically immature stage of differentiation. Further efforts to induce MHC class II antigen expression included the combination of GM-CSF with TNF-α (500 U/ml) and/or IFN-γ (1,000 U/ml) for up to 5 d and culture on a "feeder layer" of irradiated, syngeneic spleen cells. None of these treatments affected significantly the expression of cell surface I-E^k on the putative "immature" liver DC.

Allostimulatory Activity by GM-CSF–propagated Liver-derived Cells. Fig. 6 shows that, when compared with GM-CSF–stimulated spleen cells propagated and harvested using the same techniques, the cultured liver-derived cells failed to induce naive T cell proliferation. GM-CSF–stimulated, spleen-derived DC, however, which expressed higher levels of surface MHC class II antigen, were more efficient inducers of primary allogeneic T cell responses than were freshly isolated spleen cells. Furthermore, failure of the GM-CSF–stimulated putative liver DC to induce MLR contrasted with the potent allostimulatory activity of an overnight-cultured, nonadherent, low density, mature, DC-enriched population that was prepared from freshly isolated normal B10.BR mouse liver NPC, using conventional methods (3, 21) (data not shown).

Induction of MHC Class II on GM-CSF–stimulated Liver-derived Cells after Exposure to Type-1 Collagen. We also determined whether the GM-CSF–propagated putative "immature" liver DC might undergo maturation when allowed to propagate in contact with extracellular matrix protein (type-1 collagen) that is expressed constitutively in the local microenvironment

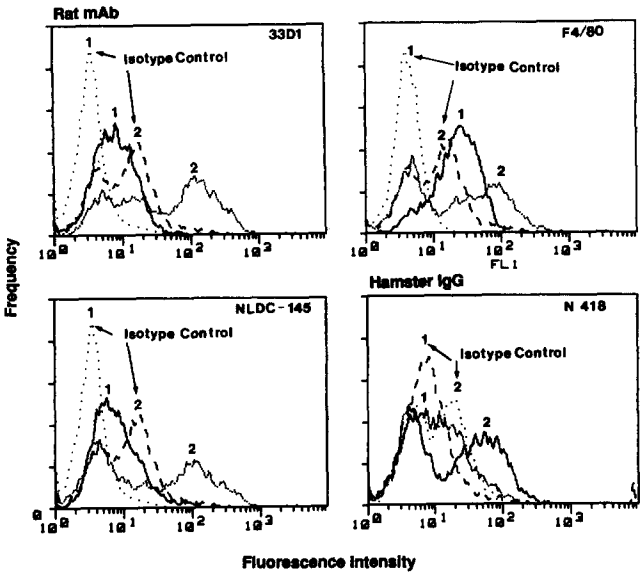

Figure 7. The expression of various DC-restricted markers and of F4/80 on GM-CSF–stimulated putative liver DC before (*1*) and after (*2*) the exposure of the cells to type-1 collagen. 7-d cultures of liver-derived cells released from aggregates in GM-CSF–supplemented medium were exposed for a further 3 d to type-1 collagen or maintained without collagen in the continuous presence of GM-CSF (0.4 ng/ml). An isotype-matched irrelevant Ab was used as a negative control. The results are representative of three separate experiments.

of the liver (portal triads, stroma of the portal spaces, and around central veins) in which mature DC are most readily detected (11, 13). Day 7 GM-CSF–stimulated liver cells expressing low levels of MHC class II were transferred to culture plates precoated with type-1 collagen and maintained for three more days in the presence of GM-CSF. Cell proliferation was observed on the collagen-coated plates, accompanied by a relative increase in nonadherent cells as compared with control cultures (collagen free). Immunophenotypic analysis of the nonadherent cells showed marked upregulation in the intensity of expression of the DC markers NLDC145, 33D1, and N418 (Fig. 7). Such upregulation of DC markers has been shown previously in GM-CSF–stimulated mouse bone marrow cultures (16). Of particular interest, however, was the marked upregulation of MHC class II expression observed on liver DC propagated for an additional 3 d on collagen-coated plates as compared with similar cells maintained in collagen-free cultures. This observation was confirmed by immunocytochemical staining of cytospins of these cells (Fig. 8, *a* and *b*). To address the possibility of enrichment of class II^+ cells, vs upregulation of class II in collagen and GM-CSF cultures, an attempt was made to deplete all of the Ia^+ population by complement-mediated lysis before exposure to collagen. Similar to the 7-d GM-CSF propagated DC, the Ia^- population obtained from these cells also markedly upregulated its MHC class II expression when exposed to collagen for 3 d in the continued presence of GM-CSF (Fig. 8, *c* and *d* and Fig. 9). Additionally, cell proliferation was also observed as $\sim 2.5 \times 10^6$ and 1.5×10^6 nonadherent cells, respectively, per well, were recovered 3 d after seeding 10^6 Ia^- cells into collagen-coated or collagen-free wells. The number of adherent cells was inversely related to the number of nonadherent cells recovered from the wells. Furthermore, the intensity of this MHC class II expression was similar to or greater than that observed on GM-CSF–stimulated spleen-derived DC (day 10) which also expressed high levels of Ia with or without exposure to the extracellular matrix protein (Fig. 8 *e*). To control for possible trace contamination of the collagen with endotoxin and to rule out possible endotoxin-mediated upregulation of class II on the developing liver DC, 7-d cultures were exposed to LPS (50 μg/ml) for a further 3-d period in the presence of GM-CSF. No increase in class II expression was observed.

Reduction in Phagocytic Activity after Exposure of Developing Liver LC to Type-1 Collagen. We compared the ability of the developing, liver-derived DC to phagocytose opsonized SRBC before and after 3-d exposure of the cells to type-1 collagen in the continued presence of GM-CSF. In two separate experiments, after exposure to collagen, and with concurrent upregulation of MHC class II expression, the liver-derived DC showed a marked reduction in erythrophagocytic activity (Fig. 10).

Development of MLR Stimulatory Activity after Exposure of Developing Liver DC to Type-1 Collagen. After exposure to collagen, Ia-depleted liver DC became potent inducers of MLR, in marked contrast to Ia-depleted cells maintained in GM-CSF alone, which failed to elicit T cell proliferation (Fig.

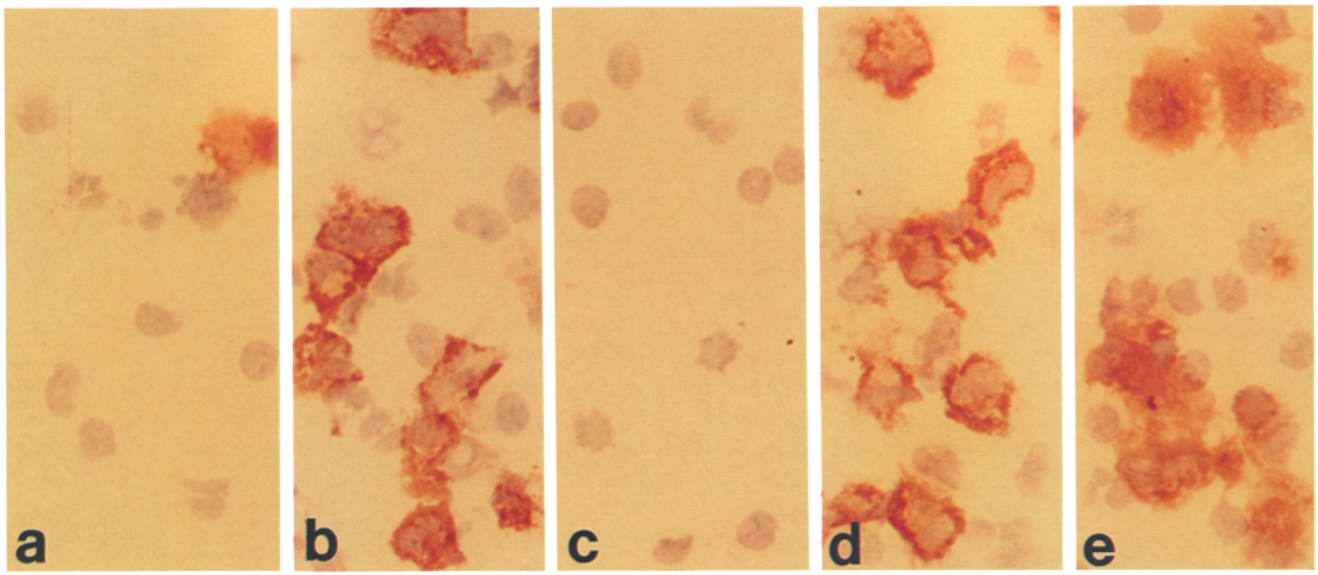

Figure 8. Immunoperoxidase staining of cytocentrifuge preparations of GM-CSF–stimulated liver- or spleen-derived cells for MHC class II (I-E^k) antigen. (*a*) A very low level of MHC class II expression was found on liver-derived putative DC released from aggregates after 7 d in culture. (*b*) After an additional 3-d exposure to type-1 collagen in the continued presence of GM-CSF, I-E^k expression on liver cells was greatly increased. (*c*) Few class II^{dim} cells from (*a*) were depleted by anti-Ia mAb and complement. (*d*) After 3-d exposure to collagen type-1 in the continued presence of GM-CSF, the expression of class II was markedly upregulated. (*e*) GM-CSF–stimulated spleen-derived DC, which were propagated and harvested (day 10) under similar conditions in the absence of collagen and which showed a high level of MHC class II expression. ×600.

11). These class II^{bright} liver-derived DC also proved much stronger MLR stimulators than freshly isolated spleen cells, although not as potent as GM-CSF–stimulated splenocytes.

Homing of Liver-derived DC. To assess the homing ability of the developing liver DC propagated in culture, 10-d GM-CSF–stimulated cells (1 or 2.5 × 10^5 low I-E^k expression or Ia^- after complement-mediated lysis, respectively) were injected subcutaneously into one hind footpad or intravenously into allogeneic B10 (I-E^-) recipients. For comparative analysis, strongly class II^+ GM-CSF–stimulated spleen DC (10-d cultures) were injected into separate animals. 1–5 d later, the animals were killed and cryostat sections of the draining lymph nodes (where appropriate) and spleens were stained with donor-specific mAb to I-E^k. As shown in Fig. 12, liver-derived cells propagated in GM-CSF–supplemented cultures homed after injection almost exclusively to the T cell areas

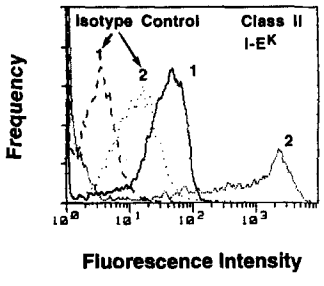

Figure 9. Flow cytometric analysis of the expression of MHC class II (I-E^k) on GM-CSF–stimulated putative liver DC with or without subsequent exposure to type-1 collagen. Ia^+ cells were depleted from 7-d cultures of liver-derived cells released from aggregates in GM-CSF–supplemented medium by treatment with anti-Ia (I-E^k) mAb and complement. The cells were then exposed for a further 3 d to type-1 collagen (*1*) or maintained without collagen (*2*) in the continuous presence of GM-CSF (0.4 ng/ml). An isotype-matched irrelevant Ab was used as a negative control. The results are representative of three separate experiments.

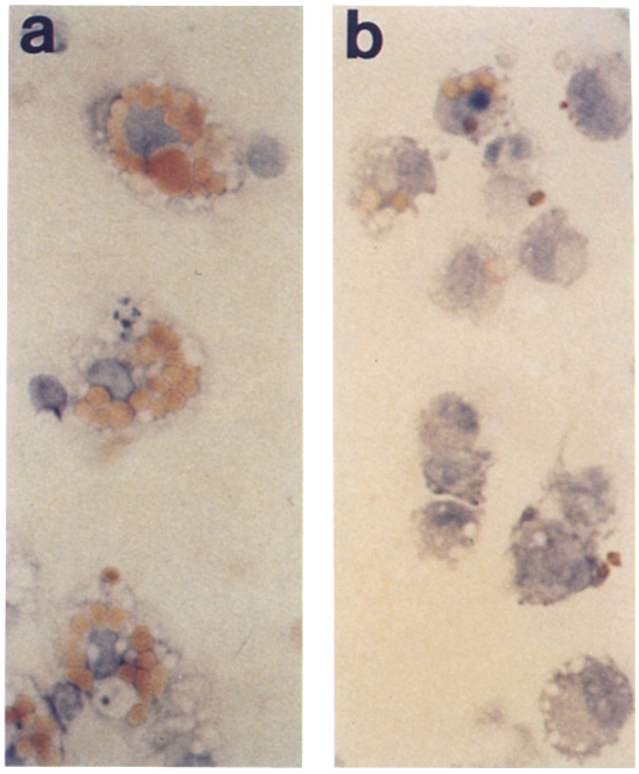

Figure 10. Exhibition of phagocytic activity by GM-CSF–stimulated, liver-derived DC before and after culture in the presence of type-1 collagen. (*a*) Cells released from aggregates 7 d after the initiation of liver cell cultures showed avid phagocytic activity after exposure for 2 h at 37°C to SRBC opsonized with a subagglutinating concentration of rabbit IgG antibody. In contrast (*b*), liver-derived DC cultured for a further 3 d on collagen-coated plates in the continued presence of GM-CSF showed markedly reduced phagocytic activity. Giemsa ×400.

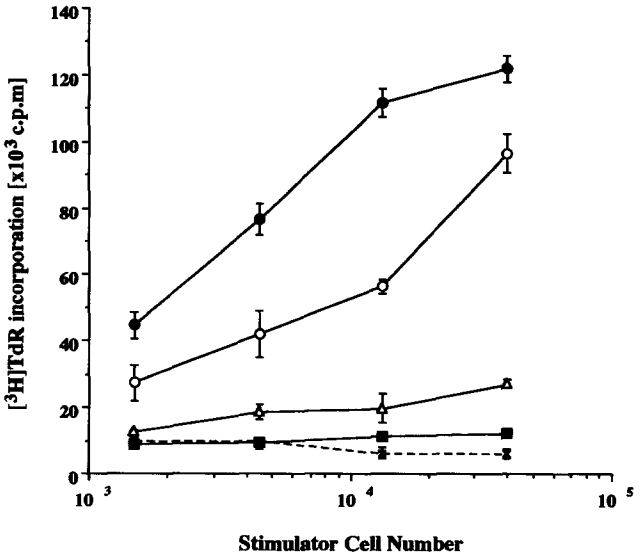

Figure 11. Allostimulatory activity of γ-irradiated, GM-CSF–stimulated B10.BR liver-derived DC from which all class II$^+$ cells were depleted (after 7-d culture) with anti-Ia mAb and complement; the cells were then exposed to type 1-collagen for 3 d. (■) Ia-depleted stimulator cells maintained for 3 d with GM-CSF in collagen-free wells; (○) similarly derived and treated cells, except for exposure for 3 d to type-1 collagen with continued presence of GM-CSF. There was no increase in the MLR stimulatory activity of untreated or Ia-depleted liver-derived cells maintained in GM-CSF alone for extended periods beyond 7 d (up to 35 d; data not shown). The allostimulatory activity of freshly isolated (△) and GM-CSF–stimulated (day 10) B10.BR spleen-derived DC (●) and (X) syngeneic (B10) spleen cells is also shown. The results are expressed as mean counts per minute ± 1 SD.

of recipients' spleens in close proximity to arterioles. Similar observations were also made in the draining lymph node of footpad-injected mice (data not shown). Moderate to intense I-E^k expression was detected on the liver-derived cells, many of which also exhibited distinct dendritic morphology. At day 5 after injection, liver-derived DC in the recipient's spleen were more abundant than strong class II$^+$ spleen-derived DC, which also homed after injection to T cell areas of recipients' spleens and lymph nodes (data not shown). Similar observations were made whether Ia^{dim} or Ia-depleted cells were injected. In the latter instance, however, the incidence

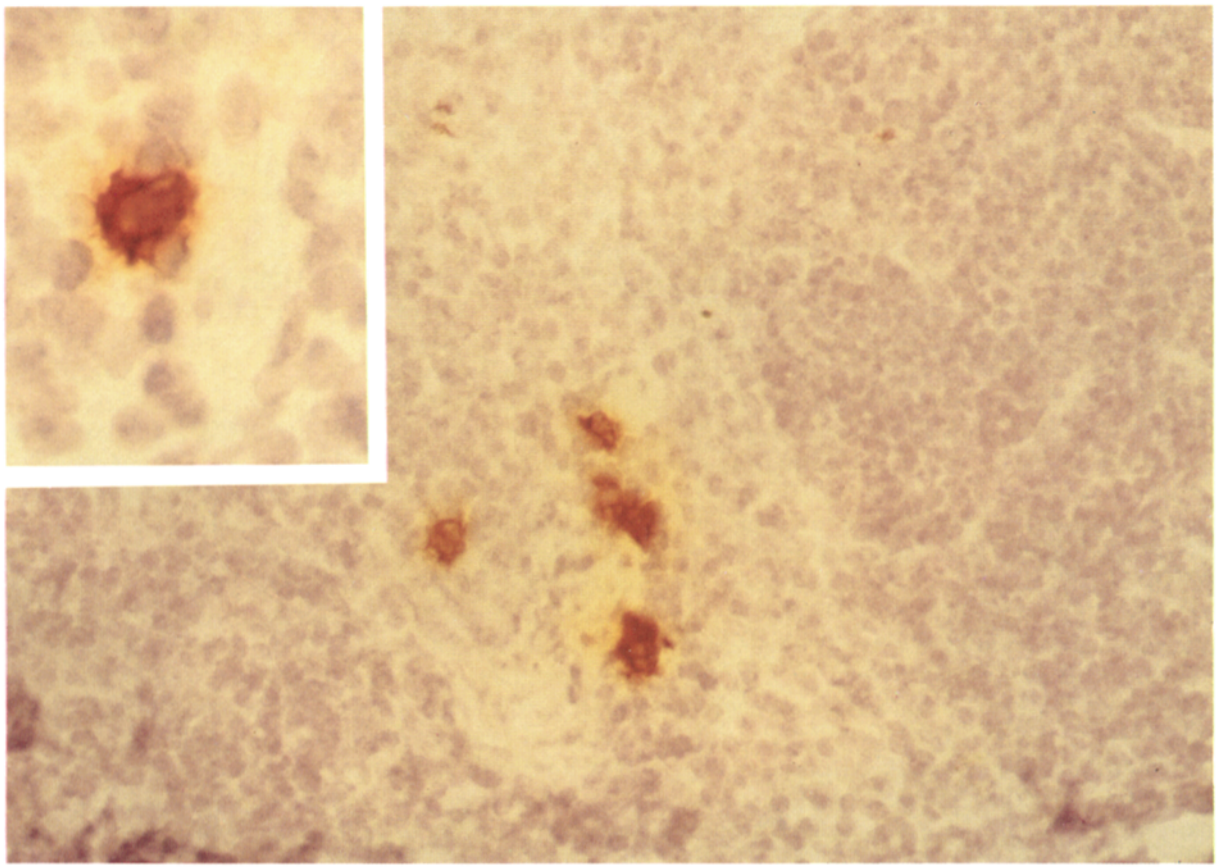

Figure 12. Homing ability of GM-CSF–stimulated B10.BR liver-derived cells (I-E^{dim}) released in culture from proliferating cell aggregates. The cells (1 or 2.5 × 10^5) were injected subcutaneously into one hind footpad of B10 (I-E$^-$) recipients and detected by immunohistochemistry in cryostat sections of draining lymph nodes and spleen 1–5 d later. The spleen sections were stained using the ABC peroxidase procedure with donor-specific mouse anti-I-E^k mAb or appropriate controls. Strongly MHC class II–positive cells were readily identified in the T-dependent areas often in close proximity to arterioles. ×400. Similar results were obtained when animals were injected with cells that were depleted of all of the class II$^+$ population with anti-Ia mAb and complement, although the numbers of I-E^{k+} cells detected in the recipients' tissues were significantly reduced. (*Inset*) Dendritic morphology is evident in a high-power view of a donor I-E^{k+} liver-derived cell in the recipient's spleen. ×1,000.

of positive cells was reduced. These observations suggest that the immature liver-derived DC after injection upregulate their MHC class II surface antigen in vivo.

Discussion

It has been shown previously in mice that orthotopic B10.BR ($I\text{-}E^k$) hepatic allografts survive permanently when transplanted into nonimmunosuppressed B10 ($I\text{-}E^-$) recipients (MHC class I and II disparate) and induce donor-specific acceptance of skin and cardiac grafts (10). In the present studies, cultured liver NPC and splenocytes from B10.BR mice were examined. The myeloid lineage cells propagated selectively from the spleen in the presence of GM-CSF had the morphologic features, cell surface immunophenotype, allostimulatory activity, phagocytic capacity, and homing characteristics classically ascribed to DC (5).

Unlike DC cultured from the spleen, however, the majority of GM-CSF–stimulated NPC derived from the liver had weak or no surface expression of MHC class II and failed to stimulate naive B10 T-lymphocytes in MLR. Consequently, their identity as DC could not be established unequivocally. This dilemma was resolved by culturing the liver cells in the presence of type-1 collagen, a strategy employed to mimic the microenvironment of the intact liver, where mature DC are found in areas that are rich in type-1 collagen, i.e., portal tracts, around central veins, and in Glisson's capsule (17). After 3 d of exposure to type-1 collagen in the presence of GM-CSF, the putative hepatic DC markedly upregulated the expression of MHC class II with concomitant reduction in their phagocytic capacity and acquisition of potent allostimulatory activity. These observations could reflect selective "enrichment" after exposure to collagen of few Ia^{dim} cells, rather than the functional and phenotypic maturation of liver DC precursors. This argument however, was dismissed after our findings that Ia^- GM-CSF–propagated liver DC, when exposed to collagen, also matured into potent APCs expressing equally high levels of MHC class II.

Evidence that the foregoing lineage of hepatic NPC can exhibit the homing capacity characteristic of DC was obtained by injecting class II^{dim} or class II^- cells subcutaneously into the footpad or intravenously into normal B10 mice. When the recipients were killed after 1–5 d, the injected cells had homed to the draining lymph node and spleen, where they were easily identified as donor in T cell areas by their moderate to dense MHC class II ($I\text{-}E^k$) expression.

These findings are consistent with the key role we have ascribed to the DC in the migration of bone marrow–derived cells from whole organs after their transplantation, resulting in low level chimerism and donor-specific immunologic nonreactivity (1, 2, 10). We have postulated recently that cell migration and chimerism may be the basis for the acceptance of all allografts (1, 2, 7). The unusual tolerogenicity of the liver could mirror a comparatively heavy content within this organ of immature or progenitor DC (22), that was shown in this study to rapidly disseminate and upregulate their expression of MHC class II in allogeneic recipients. Furthermore, work in our laboratory has shown that using GM-CSF, the propagation of myeloid lineage cells from normal mouse heart NPC is much more difficult to achieve. It has been suggested from observations in rats (6) and humans (7), and demonstrated unequivocally in mouse liver transplant experiments (10), that multiple hematolymphopoietic lineages participate in the leukocyte traffic that occurs after organ transplantation. These lineages may survive permanently without treatment in mice (10) or after an induction course of immunosuppression in rats (6) and even in some humans (7). The probability that there are pluripotent stem cells and immature DC (among other lineages) in the bone marrow–derived interstitial population of nonlymphoid organs is inherent in our cell migration and chimerism hypothesis (1, 2).

The survival and maturation of liver DC progenitors that home to T cell–dependent areas of lymphoid tissue in the allogeneic recipients herein reported is consistent with the contention of Demetris et al. (6) and Qian et al. (10) that the destination of the cell, trafficking after whole organ transplantation, is lineage specific, following the same routes taken by syngeneic cells of the same lineages. After liver and other organ transplantation, immature cells of DC lineage may undergo in vivo maturation with endogenous GM-CSF and other cytokines in an in vivo recapitulation of the cell culture experiments of Romani et al. (23), and Inaba et al. (15, 16), and of the in vitro experiments reported herein. The extent and tempo of this maturation process are undoubtedly influenced by the level of host immunosuppression when such treatment is used. However, all of our in vivo experiments were performed in the absence of therapy or host preconditioning.

There are several lines of evidence that complex interactions between donor leukocytes and the recipient's immune system can lead to decreased host alloresponsiveness and even to tolerance induction (1, 2, 6, 7, 24–27). There are also numerous reports that mature T cells can be tolerized to allogeneic antigens outside the thymus (28, 29). It is therefore plausible that chimeric cells present in the periphery may play a key role in achieving allotolerance. The tolerogenic implications of the rapid egress from transplanted whole organs (the liver above all) of postulated precursor or progenitor DC exhibiting low MHC class II expression and low T cell–stimulating activity are considerable. The existence of subpopulations of murine DC with a veto function has recently been proposed (30). Moreover, $HLA\text{-}DR^{dim}$ allogeneic donor bone marrow cells, shown to exhibit veto cell activity (inactivation of T helper cells or cytotoxic T cell precursors) have been postulated to be immature DC (31). Such immature DC, which were shown in our experiments to have avid phagocytic activity in culture, might be expected to elicit a "deviant" (tolerogenic) local and systemic immune response shortly after their injection. The precise basis of the DC–T cell interaction leading to tolerance induction is uncertain, but would clearly depend on the relative affinity or avidity (compared with effective APC) of the donor DC–TCR interactions and on the expression on the former cells, of adhesins

and costimulatory molecules, such a B7/BB1. These aspects of the developing liver-derived DC are under further investigation in our laboratory.

A well-studied example of an atypical or deviant cytokine-modulated immune response induced by bone marrow–derived APC has been provided by Wilbanks and Streilein (32). They have studied class II^{dim} APC with dendritic morphology which are now thought to be variant DC in the iris, ciliary body, and other tissues lining the anterior chamber of the eye (33, 34). When BSA was injected into the anterior chamber and taken up by these APC, it was presented ineffectively both to the T cells locally, and within a few hours, in the spleen, after the arrival there of the APC–peptide complexes. The consequence was a dampened systemic as well as local (ocular) immune response to a subsequent challenge with the antigen.

The exploitation of putative tolerogenic DC for the induction of controlled transplantation tolerance as a consequence of donor cell chimerism is not hard to imagine. Although DC precursors apparently are concentrated in the liver, a similar subpopulation presumably may exist in all other tissues and whole organs and particularly in the bone marrow, which is the origin of the leukocyte population of the liver and elsewhere. The clinical implications of these observations are far-ranging, prompting us to initiate a more thorough investigation of DC precursors in bone marrow, spleen, and other tissues.

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