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Phenotypic and Functional Characterization of Mouse Hepatic CD8α+ Lymphoid-Related Dendritic Cells

Peta J. O’Connell,* Adrian E. Morelli,* Alison J. Logar,* and Angus W. Thomson²*†

Recently, attention has focussed on phenotypic and functional differences between classic myeloid dendritic cells (DC), and DC that reportedly develop from an early, committed lymphoid precursor. In mice, DC from these separate hemopoietic lineages differ by their surface expression of CD8α. We undertook a comparative study of CD8α+ (CD11bhigh; myeloid)- and CD8α− (CD11blow; lymphoid-related) and CD8α− (CD11bhigh; myeloid) DC isolated from mouse liver. CD8α+ and CD8α− DC each constituted ≤1.0% of the freshly isolated, normal nonparenchymal cells (NPC). Both populations were enriched 10–15% by overnight culture and metrizamide density centrifugation. Flt3 ligand (Flt3L) potently induced equal expansion of both subsets in vivo. Tissue-resident CD8α+ DC, both populations were enriched 10–15% by overnight culture and metrizamide density isolated from Flt3L-treated mice, existed primarily as immature cells (CD11c+, CD11blow, CD40low, CD80low, CD86low, MHC class II+), consistent with previous observations regarding bulk DC freshly isolated from nonlymphoid tissues. Following overnight culture in GM-CSF, CD8α+ DC underwent phenotypic and functional maturation equivalent to that observed for CD8α− DC. CD95 ligand (FasL) mRNA was detected in both immature and mature DC of each subset. In vitro analysis confirmed that flow-sorted, mature CD8α+ and CD8α− DC were strong and equally efficient stimulators of allogeneic T cell proliferation in primary MLR. Both immunohistochemical and genomic DNA analysis revealed that in vivo, sorted CD8α+ DC trafficked from s.c. sites to T cell areas of allogeneic lymphoid tissue and were equally efficient at priming naive T cells compared with CD8α− DC. This is the first comparative study of lymphoid-related DC isolated from nonlymphoid tissue. The Journal of Immunology, 2000, 165: 795–803.

Dendritic cells (DC) are rare, bone marrow-derived leukocytes, uniquely specialized for immunosurveillance and the activation of lymphocytes (1). DC are found as immature cells, in the interstitium of virtually all organs and tissues, where they have the ability to capture and process foreign and self Ag. During this process, DC migrate to secondary lymphoid tissues, where they present antigenic peptides to naive, Ag-specific T cells. Considerable new information concerning the ontogeny of DC, the factors that regulate their maturation and tissue distribution, and the role of these important APC in the induction and regulation of immune responses has accumulated over the past several years. This information has been extensively reviewed (2–5).

Until recently, DC were thought to develop directly from a CD34+ progenitor or from a committed myeloid lineage precursor. GM-CSF, IL-3, and Flt3 ligand (Flt3L) have been demonstrated to promote the growth and differentiation of these DC progenitors, while TNF-α and CD40L induce functional maturation of myeloid DC (MDC) (2–5). It has become increasingly apparent that DC also develop from committed lymphoid precursors in the absence of erythroid or granulocyte-macrophage lineage differentiation. Reconstitution of irradiated mice (7.5 Gy) with thymic lymphoid precursors (CD4+low, CD44+high, CD90+low, MHC class II+) results in DC maturation, concurrent with the development of T, B, and NK cells (6), while the T lineage-committed precursor (CD4−, CD8−, CD25−, CD44+) gives rise to DC and T cell development only (7). In vitro culture of these T lineage-leukocyte-committed precursors (8) or, alternatively, CD19+ pro-B cells (9) with a complex cytokine cocktail (TNF-α, IL-1, IL-3, IL-7, c-Kit ligand, CD40L, and Flt3L) results in DC development. Interestingly, GM-CSF does not appear to be necessary for DC differentiation from committed lymphoid precursors, either in vitro or in vivo (8, 10, 11); however, it is reported to improve bulk spleen-derived DC survival and recovery in short term culture (12). Lymphoid-related DC (LDC) express the DC-associated Ags CD11c and DEC-205, MHC class II, and costimulatory molecules (CD40, CD80, CD86), but lack, or express at low levels, the myeloid-associated β2 integrin CD11b/CD18 (Mac-1), typically expressed by MDC. Unlike MDC, the T lineage LDC isolated directly from mouse tissues also expresses CD8, mostly as an αα homodimer, although low levels of the αβ heterodimer have also been detected (12). A human DC of lymphoid origin was first described by Galy and colleagues (10). In vitro culture of bone marrow precursors (CD45RA+, CD10+, CD90high) gives rise to T, B, and NK cells and DC in the absence of myeloid differentiation, while the plasmacytoid cells (CD4+), CD3−, CD11c−) isolated from both peripheral blood and tonsil develop into distinctive DC following culture with IL-3 (13–15). Whether these mouse and human LDC represent similar DC subsets remains to be elucidated.

CD8α+ DC have been identified previously in a number of lymphoid and nonlymphoid tissues, including bone marrow, peripheral blood, spleen, thymus, lymph node, lung, and liver, from both normal and Flt3L-treated mice (7, 16, 17). DC isolated from mice treated with Flt3L are phenotypically and functionally similar to

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3 Abbreviations used in this paper: DC, dendritic cells; LDC, lymphoid-related dendritic cells; MDC, myeloid dendritic cells; Flt3L, Flt3 ligand; CD40L, CD40 ligand; NPC, nonparenchymal cells; ABC, avidin-biotin complex; Ps, peroxidase; AP, alkaline phosphatase; PALS, periaortic/iliac lymphatic sheath.
those isolated or cultured from normal animals (17–20). Here, we consider the phenotypic characteristics and functional properties of hepatic CD8α+ DC, both in vitro and in vivo. Liver interstitial DC may play important roles in host responses to infectious agents, in autoimmune and malignant diseases, and in the regulation of immune responses to liver allografts (21) that exhibit tolerogenic properties in various species (22–26). Although phenotypic and functional properties of liver DC have been described, attention to date has focussed almost exclusively on MDC. There has been considerable speculation regarding the potential tolerogenic capacity of CD8α+ DC (27) arising from observations that in vitro they regulate the proliferation of CD4+ and CD8+ T cells via CD95 (Fas)-CD95L-dependent, activation-induced cell death (28), or by limiting IL-2 production (29), respectively. The present report is the first detailed comparative study of CD8α+ DC isolated from a nonlymphoid tissue and considers the kinetics of the induction of these cells by Flt3L in the liver. We have identified both immature and mature CD8α+ DC populations. Highly purified, sorted hepatic CD8α+ DC were compared directly with CD8α− DC for their Ag-presenting function, their homing ability, and their capacity to prime allogeneic T cells in vivo.

Materials and Methods

Animals

Male C57BL/10 (B10; H2b) and C3H/HeJ (C3H; H2b) mice, 8–12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in the specific pathogen-free facility of the University of Pittsburgh Medical Center and were provided with Purina rodent chow (Ralston Purina, St. Louis, MO) and tap water ad libitum.

Reagents

FITC-, PE-, or Cy-Chrome-conjugated mAbs to detect cell surface CD3ε, CD8α, CD10, CD11b, CD11c, CD40, CD80, CD86, and IAbβ chain expression by flow cytometry were purchased from PharMingen (San Diego, CA). Purified rat anti-CD8α (53-6,7), hamster anti-CD11c (HL3), biotin-conjugated hamster anti-CD3ε (145-2C11), and mouse anti-IAββ chain (25-9-17) mAb (PharMingen) were used for immunostaining of cytopsins and tissue sections. Recombinant mouse GM-CSF was provided by the Immunex Research and Development Corp. (Seattle, WA). RPMI 1640 (Life Technologies, Gaithersburg, MD) was supplemented with 10% FCS (Nalgene, Miami, FL), nonessential amino acids, L-glutamine, sodium pyruvate, penicillin-streptomycin, and 2-ME (all from Life Technologies) and is referred to subsequently as complete medium.

DC isolation and sorting

DC were isolated from the livers of animals given Flt3L (10 μg mouse/day i.p. in HBSS) for 6–10 consecutive days. Liver NPC were isolated as described previously (30), with the following modifications. Briefly, the liver was perfused in situ with 30 ml of HBSS, followed by 5 ml of type IV collagenase (1 mg/ml; Sigma) in HBSS. The liver was then removed, teased apart, and digested for an additional 30 min with 20 ml of the collagenase solution at 37°C. The resulting mixture was resuspended with 5 ml of HBSS and further purified by centrifugation using 30% metrizamide at 1200×g for 20 min at 4°C. NPC were incubated overnight (18 h) in RPMI 1640 complete medium. The resulting mixture was resuspended with complete medium. Recombinant mouse GM-CSF was provided by Immunex Research and Development Corp. (Seattle, WA). RPMI 1640 (Life Technologies, Gaithersburg, MD) was supplemented with 10% FCS (Nalgene, Miami, FL), nonessential amino acids, L-glutamine, sodium pyruvate, penicillin-streptomycin, and 2-ME (all from Life Technologies) and is referred to subsequently as complete medium.

Mixed leukocyte reaction

Single-cell preparations from lymph nodes or spleens of C3H mice were used as responders. Splenocytes were enriched for T cells by a single passage through nylon wool columns (45 min; 37°C). Cells (2×10⁷) were placed in each well of 96-well, round-bottom plates, and varying numbers of gamma-irradiated (20 Gy), sorted, overnight-cultured CD8α− or CD8α+ DC were washed extensively in HBSS, then injected s.c. (1–2.5×10⁵ in 50 μl) into the footpad or flank of one hindlimb. The regional lymph nodes (popliteal and inguinal), spleen, and thymus were removed after 2 days for immunohistochemical and molecular analyses and after 7 days for functional studies (lymph node only). Trafficking DC were identified by staining for donor MHC class II (IAβ; see below) and by detection of genomic DNA.

Immunostaining of tissue sections

Tissue samples were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap-frozen in isopentane (prechilled in liquid nitrogen), and stored at −80°C until use. Eight-micron cryostat sections were mounted on slides treated with Vectabond (Vector), air-dried, and fixed in cold acetone, 1% osmium tetroxide/1% FeCl₃ for 1 h, followed by dehydration through a graded ethanol series (30–100%). Pellets were then embedded in Polybed 812, and ultrathin sections (60 nm) were obtained using a Reichert Ultracut E microtome (Vienna, Austria). Sections were poststained in 2% uranyl acetate in 50% methanol for 10 min, and 1% lead citrate for 7 min and viewed on a JEOL JEM 1210 electron microscope (Peabody, MA) at 80 kV.
2). Although the total number of leukocytes in bone marrow and treated animals, including bone marrow, spleen, and thymus (Fig. CD8 To determine the time course of Flt3L-mediated induction of leukocyte fraction (Fig. 1 CD8 were substantially enriched to 12–14% of the low buoyant density subset. Flt3L increased the total number of CD8 and CD11b CD subsets. Flt3L increased the total number of CD8 and CD11b CD subsets. Flt3L increased the total number of CD8 and CD11b CD subsets. Flt3L increased the total number of CD8 and CD11b CD subsets. Flt3L increased the total number of CD8 and CD11b CD subsets. Flt3L increased the total number of CD8 and CD11b CD subsets. Flt3L increased the total number of CD8 and CD11b CD subsets. Flt3L increased the total number of CD8 and CD11b DC frequency was increased 15-fold by Flt3L administration. The progressive increase in total CD8 and CD11b DC in the liver is shown in Fig. 1B. Both DC subsets were increased relatively equally by Flt3L. CD8α+ DC could readily be identified in other tissues of Flt3L-treated animals, including bone marrow, spleen, and thymus (Fig. 2). Although the total number of leukocytes in bone marrow and the thymus was not altered substantially by Flt3L administration, small increases in CD8α+ DC frequency were noted. In contrast, the total number of splenocytes was increased 5-fold (data not shown), and the CD8α+ DC frequency was increased 15-fold by

Genomic PCR

Genomic DNA was extracted as described previously (33) with the following modifications. Tissue samples were digested overnight in lysis buffer (0.1 M Tris (pH 8.50), 100 mM NaCl, 5 mM EDTA, and 0.2% SDS) containing protease K (1 mg/ml) at 55°C. Genomic DNA was extracted with Tris-EDTA-saturated phenol/chloroform/isoamyl alcohol (25/24/1), followed by precipitation with 3 M sodium acetate (pH 5.2) in isopropyl alcohol. Excess salt was removed by washing with 70% ethanol. DNA was air-dried briefly and resuspended in Tris-EDTA. One microgram of genomic DNA was used for each PCR, and relatively equal quantities were ensured by amplifying β-actin as described for RT-PCR above. Donor-specific signal was detected by amplification of donor MHC class II (IAa) DNA in the C3H recipients as previously described (31). Genomic DNA from B10 (0.1 μg in 0.9 μg of C3H) and C3H splenocytes were used as positive and negative controls, respectively.

Results

CD8α+ and CD11b+ (CD8α−) DC in normal liver and kinetics of Flt3L-induced DC generation

The frequency of both CD8α+ and CD11b+ (CD8α−) DC in freshly isolated, normal liver NPC (day 0) was determined to be very low (<1%; Fig. 1A), which represents the lower limit of accurate flow cytometric detection. To convincingly demonstrate the presence of both DC populations in normal liver, well-established protocols for DC enrichment (overnight incubation of the bulk leukocyte population, followed by metrizamide density centrifugation) were used. Both CD8α+ and CD11b+ (CD8α−) DC were substantially enriched to 12–14% of the low buoyant density leukocyte fraction (Fig. 1A), thus confirming that each subset was present in normal liver and in approximately equivalent numbers. To determine the time course of Flt3L-mediated induction of CD8α+ and CD11b+ (CD8α−) DC in the liver, mice were injected with 10 μg of Flt3L once daily for 6–10 days, a regimen previously described for the optimal induction of DC subsets in mouse lymphoid and nonlymphoid tissues (17, 34). At the end of each treatment period, NPC were isolated as described in Materials and Methods, immunolabeled, and analyzed by flow cytometry. Flt3L administration substantially increased the frequency of DC11c+ cells to a maximum of 55–60% of the total NPC population, which was maintained from days 8–10 of treatment (Fig. 1A). Similar increases were observed in the CD8α+ (29%; day 10) and CD11b+ (27%) DC subsets. Flt3L increased the total number of DC per liver most dramatically, with the maximal increase of 650-fold observed after 10 days of treatment. The progressive increase in total CD8α+ and CD11b+ DC in the liver is shown in Fig. 1B. Both DC subsets were increased relatively equally by Flt3L. CD8α+ DC could readily be identified in other tissues of Flt3L-treated animals, including bone marrow, spleen, and thymus (Fig. 2). Although the total number of leukocytes in bone marrow and
Flt3L administration (Fig. 2). These data regarding bone marrow, thymus, and spleen are consistent with previous reports (17, 35).

Morphologic appearance of liver CD8α+ DC

Double immunostaining to detect coexpression of CD8α and CD11c (LDC), identified a small number of DC-like single cells in the portal spaces and the perivenular regions of normal mouse livers (Fig. 3A). Other cells with dendritic morphology but CD8α− CD11c+ (myeloid DC), and small round CD8α− CD11c− cells (presumed CD8 T cells) were also detected (Fig. 3A). CD8α+ CD11c+ liver DC induced by Flt3L were examined morphologically both in cytocentrifuge preparations and by transmission electron microscopy of flow-sorted specimens. As shown in Fig. 3, B–D, these cells exhibited typical reniform or lobulated nuclei, few prominent granules or vesicles, and extensive cell membrane projections characteristic of DC, including liver-derived MDC (30). Birbeck granules were not observed.

Phenotype and purification of liver-derived DC subsets

Consistent with previous reports (12) regarding thymic and splenic DC, both freshly isolated and overnight-incubated, hepatic CD8α+ DC expressed low levels of CD11b (Fig. 4A), in contrast to the high levels detected on CD8α− DC (Fig. 4B). There was a broad gradation of CD8α expression by DC, and the mean fluorescence intensity of CD8α expression was generally less on DC compared with that on CD11c+ CD8α− hepatic T cells (Figs. 1A and 5A). Interestingly, a small (14–20%) subset of CD8α− DC coexpressed low levels of surface CD8β (Fig. 4C). Vremec et al. (12) reported low levels of expression of the CD8αβ heterodimer by thymic DC, indicating that the αα homodimer was the main form of CD8 expression on LDC.

To further examine the surface Ag phenotype of DC subsets isolated from Flt3L-treated mouse liver, NPC were triple immunostained, both when freshly isolated and following overnight (18-h) incubation in the presence of GM-CSF. GM-CSF has been reported to improve bulk DC viability and recovery in short term splenocyte cultures (11). Inclusion of GM-CSF during overnight incubation improved the survival and recovery of NPC, assessed by uptake of the nonvital stains PI or trypan blue with detection by flow cytometry or direct cells counts, respectively. Typically, NPC viability and recovery were improved 12–25% by inclusion of GM-CSF during overnight culture (data not shown).

In freshly isolated liver NPC, both DC subpopulations harvested after 10 days of Flt3L administration expressed a surface phenotype of immature DC (Fig. 5A). Thus, low levels of expression of MHC class II (IAα), CD80, and CD86 were detected, while staining for CD40 was low/negative. Interestingly, CD8α+ DC from freshly isolated NPC expressed consistently higher levels of MHC class II, CD80, and CD86 (four experiments), with a minor population (∼10%) of CD40+ cells. By contrast, after overnight culture in GM-CSF, both CD8α+ and CD8α− DC markedly up-regulated expression of these Ags to become MHC class IIhigh, CD40high, CD80high, and CD86high (Fig. 5B). The expression of MHC class II and costimulatory molecules on both subsets cultured overnight was substantially reduced in the absence of GM-CSF (Table I). However, the relative levels of MHC class II and costimulatory molecule expression between CD8α+ and CD8α− DC subsets were similar in both the presence and the absence of GM-CSF. In subsequent experiments CD8α+ and CD8α− DC subsets were sorted using gates to exclude CD11cdim, low forward/side scatter ratio cells, which generally included a high proportion of dead or dying cells.

RT-PCR analysis of CD95L (FasL) expression

The expression of gene transcripts for CD95L in both freshly isolated and overnight-cultured, sorted CD8α+ and CD8α− DC from the liver was identified using PCR (Fig. 6). In two separate experiments, transcripts for CD95L were identified in all DC subpopulations, with the highest level detected in freshly isolated, immature CD8α+ DC in both experiments. Expression of cell surface...
CD95L protein was not detected by mAb staining and flow cytometry on either freshly isolated or overnight-cultured CD8α1 or CD8α2 DC (data not shown).

Comparative allostimulatory activity of sorted CD8α1 and CD8α2 DC in vitro

The ability of sorted, overnight-cultured B10 CD8α+ and CD8α- DC to stimulate the proliferation of naive allogeneic (C3H) T cells was assessed initially in vitro. As shown in Fig. 7, both DC populations were extremely efficient stimulators of allogeneic splenic T cells in primary 72-h MLR and induced almost identical degrees of proliferation. The CD8α1 and CD8α2 DC were ~10-fold more efficient compared with bulk splenocytes of the same allogeneic (B10) strain at equivalent ratios of stimulators and responders.

Trafficking of sorted liver CD8α+ DC to lymphoid organs of allogeneic recipients

To examine the ability of CD8α+ DC to migrate from the periphery to host lymphoid tissue, sorted, overnight-cultured, CD8α+ CD11c+ cells were injected s.c. into one hind footpad of normal allogeneic (C3H) mice. Two days after injection, animals were killed, and the donor CD8α+ DC were detected in samples of draining popliteal lymph nodes, spleen, and thymus by both mAb staining and genomic PCR for donor-specific MHC class II (IAb). Association of donor DC with T cell areas was assessed by double immunostaining of tissue sections for donor MHC class II in combination with anti-CD3 mAb. On day 2 after their s.c. administration, most donor CD8α+ DC were detected in the subcapsular and paracortical sinuses of popliteal lymph nodes (Fig. 8A), with very few CD8α+ DC in the T cell areas of the paracortex. In the spleen, CD8α+ DC were located in the periarteriolar lymphatic sheaths (PALS; T cell area), with most in close proximity to the arterioles (Fig. 8B). In the thymus, rare donor CD8α+ DC were identified at the cortico-medullary junction (not shown). Donor-specific MHC class II (IAb) DNA was detected by genomic PCR in the draining popliteal lymph node (Fig. 8C), confirming migration of sorted, overnight-cultured CD8α+ DC from the periphery.

Comparative allostimulatory activity of sorted CD8α1 and CD8α2 DC in vivo

Next, the ability of sorted, overnight-cultured CD8α+ or CD8α- DC to prime allogeneic T cells in vivo was assessed. Bulk lymph node cells (combined popliteal and inguinal) from C3H mice injected s.c. in the flank of one hindlimb with either CD8α1 or CD8α2 DC and killed 7 days later were used as responders in one-way, 3-day MLRs. To a fixed number of C3H lymph node T cells, variable numbers of gamma-irradiated, normal allogeneic (B10) stimulator splenocytes were added, and the proliferative response was determined. As shown in Fig. 9, CD8α+ and CD8α- liver DC were equally efficient at priming allogeneic T cells in

**FIGURE 4.** Flow cytometric analysis of sorted hepatic DC demonstrates that CD8α+ CD11c+ DC (A) express low levels of the myeloid-associated Ag CD11b compared with the relatively high levels expressed by CD8α- CD11c+ DC (B). A minor subpopulation of CD8α+ CD11c+ DC exhibited positive staining for CD8β (C). Dotted profiles show isotype-matched control values. mfi, mean fluorescence intensity. Data are representative of three separate experiments.

**FIGURE 5.** Flow cytometric analysis of liver NPC from B10 mice after 10 days of Flt3L administration. NPC were triple immunolabeled with 1) anti-CD11c PE, 2) anti-CD8α Cy-Chrome, and 3) anti-MHC class II (IAb), anti-CD40, anti-CD80, or anti-CD86 -FITC. Scatter plots depict CD8α+ and CD8α- DC populations in freshly isolated (A) and overnight-cultured (B) NPC. Shaded profiles indicate the expression of specific markers (FITC) on freshly isolated and overnight-cultured CD8α+ and CD8α- DC subpopulations gated as shown. The data demonstrate that culture of freshly isolated, immature liver DC results in phenotypic maturation of both DC subsets. Open profiles show isotype-matched controls. The results are representative of four separate experiments.
Characterization of Hepatic CD8α⁺ DC

Table I. Expression of MHC class II and costimulatory molecules on liver DC subsets after overnight culture (18 h)

<table>
<thead>
<tr>
<th>Ag</th>
<th>CD11c⁺ DC</th>
<th>Overnight Culture⁺</th>
<th>Overnight Culture + GM-CSF</th>
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<tr>
<td>MHC class II</td>
<td>CD8α⁺</td>
<td>53</td>
<td>72</td>
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* Liver NPC were cultured overnight (18 h) in the presence or absence of GM-CSF (4 ng/ml). Three-color immunostaining and flow cytometry was used to determine the level of Ag expression on either CD8α⁺ or CD8α⁻ DC.

* Mean fluorescence intensity on an arbitrary, 1023 log channel scale.

Discussion

Few studies have reported the functional characterization of DC from the liver of any species, reflecting the difficulties inherent in isolating adequate numbers of these specialized, rare APC for ex vivo analysis. Whereas previous reports have centered on bulk DC obtained from normal (26) or Flt3L-treated (34) mouse liver or on MDC propagated in vitro from liver NPC (30), the current study examines DC lineage diversity within the liver, providing the first detailed, phenotypic, and functional characterization of hepatic CD8α⁺ (lymphoid-related) and CD8α⁻ (myeloid) DC. To obtain adequate numbers of both CD8α⁺ and CD8α⁻ liver DC for direct comparative studies, Flt3L, a potent hemopoietic growth factor, was used. Recent reports have shown that DC from the lymphoid tissues of Flt3L-treated mice exhibit similar phenotypic and functional characteristics as DC isolated or cultured from their normal counterparts (17–20). Similar to previous observations (17, 20), we have observed that Flt3L is a potent in vivo growth factor. The ex vivo proliferative responses of lymph node cells from DC-injected mice to B10 alloantigens was 5-fold greater than the level of Ag expression on either CD8α⁺ or CD8α⁻ DC. Few studies have reported the functional characterization of DC from freshly isolated NPC or of mature CD8α⁺ or CD8α⁻ DC.

Vious reports have identified low numbers of CD8α⁺ DC in multiple tissues from normal mice, including bone marrow, peripheral blood, spleen, thymus, lymph node, lung, and liver (7, 16, 17), that are elevated substantially by Flt3L administration. Lyman et al. (38) suggest that it is unlikely that Flt3L simply mobilizes existing DC from peripheral tissues, which is supported by their observations that Flt3L receptor is absent from DC, and that Flt3L induces proliferation of progenitors rather than those committed to DC lineage development. It is probable that Flt3L facilitates terminal maturation of Flt3L-sensitive progenitors in both bone marrow and other hemopoietic organs. This proposal is supported by the dramatically elevated numbers of DC detected in multiple tissues following Flt3L administration, indicating a generalized expansion and maturation of progenitors (38).

Highly purified populations of hepatic CD8α⁺ and CD8α⁻ DC were isolated reproducibly for comparative studies. As reported by others for primary and secondary lymphoid tissues, CD8 was expressed by the putative liver LDC predominantly as the αα homodimer, and its expression was related reciprocally to that of CD8α⁺. This approach to purification proved to be superior to protocols involving lineage depletion, and/or sorting on the basis of CD11b or MHC class II expression. It also provided increased yields of both DC subsets, with higher levels of purity and viability. Additionally, this approach has facilitated the sorting of immature CD8α⁻ and CD8α⁺ DC from freshly isolated NPC or of mature CD8α⁺ and CD8α⁻ DC from overnight-cultured cells. The present study reveals that both freshly isolated CD8α⁻ and CD8α⁺ DC from a nonlymphoid tissue (the liver) are immature, in keeping with previous observations on bulk DC, both in situ and freshly isolated from mouse heart or kidney (39, 40). By three-color flow cytometric analysis, we were able to show clearly that freshly isolated liver CD8α⁺ and CD8α⁻ DC from Flt3L-treated mice were predominantly immature cells (CD40⁺, CD86⁺, and MHC class II⁺), with a minor population of mature DC. A similar phenotype has been observed in vivo. The ex vivo proliferative responses of lymph node cells from DC-injected mice to B10 alloantigens was 5-fold greater than those of normal mouse lymph node cells due presumably to prior in vivo stimulation by either CD8α⁺ or CD8α⁻ DC.

FIGURE 6. RT-PCR analysis of CD95L (FasL) mRNA expression in normal B10 mouse kidney (KID; low expression), 48-h Con A-activated B10 splenocytes (SPL; high expression), and both freshly isolated immature (i), and overnight-cultured mature (m), sorted liver CD8α⁺ and CD8α⁻ DC. Amplification of β-actin was used as the internal positive control. Data are representative of two separate experiments.

FIGURE 7. Allostimulatory capacity for naïve splenic T cells (C3H) of gamma-irradiated overnight-cultured CD8α⁺ or CD8α⁻ DC isolated from B10 liver following 10 days of Flt3L administration. The data show the efficient and equivalent stimulatory capacity of sorted CD8α⁺ (▼) and CD8α⁻ (▼) DC on a per cell basis compared with that of bulk splenocytes of the same allogeneic strain (C; B10) and with that of C3H splenocytes syngeneic (●) with the responder T cells. The results were obtained from 72-h MLR and are the mean ± 1 SD from triplicate cultures. The data are representative of three separate experiments.
for CD8α+ DC freshly isolated from the spleens of Flt3L-treated mice (P. J. O'Connell et al., unpublished observations). These data contrast with earlier reports of a relatively mature phenotype for freshly isolated CD8α1 DC from the thymus, spleen, or lymph node (11, 12, 17, 41, 42). In addition to the inherent differences in maturity between DC freshly isolated from nonlymphoid and lymphoid tissue sites (1, 3, 5), the discrepancy between these previous reports and the current study may reflect differences in purification strategies. As discussed by Vremec et al. (11), the methods used in earlier investigations including lineage depletion with mAbs may preselect for mature DC. An additional confounding factor may be the use of Flt3L-treated mice in the present study. Flt3L administration elevates hemopoiesis and may increase the number of immature DC disproportionately to mature DC. However, recent reports demonstrate that both CD8α+ and CD8α− DC exhibit similar phenotypic and functional properties when isolated from either normal or Flt3L-treated mice (17–20). Future functional studies on freshly isolated immature CD8α+ and CD8α− DC will ascertain whether they exhibit properties similar to in vitro generated immature DC, which others have shown can induce Ag-specific anergy and prolong allograft survival (43, 44).

In contrast to the phenotypic characteristics of freshly isolated DC, NPC cultured overnight in GM-CSF exhibited massive up-regulation of cell surface MHC class II gene products and the costimulatory molecules CD40, CD80, and CD86. Interestingly, the overnight-cultured CD8α+ DC were virtually indistinguishable from their CD8α− DC counterparts, isolated concurrently and using the same procedures. Addition of GM-CSF enhanced spontaneous maturation of freshly isolated CD8α+ and CD8α− DC in

![FIGURE 8. Tissue “homing” of sorted, overnight-cultured B10 (H2b) liver CD8α+ DC to allogeneic C3H (H2k) recipient draining popliteal lymph node (A) and spleen (B) 2 days after s.c. administration into the hind footpad. Trafficking of CD8α+ DC to T cell areas of recipient lymphoid organs was analyzed by two-color immunohistochemistry, using anti-IAb mAb followed by ABC-AP (blue), and anti-CD3ε mAb followed by ABC-Px (red) as described in Materials and Methods. On day 2, CD8α+ DC (blue; arrows and inset) were located in the lymph nodes, mainly in the subcapsular and paracapsular areas. Only a few of the CD8α+ DC were identified in the paracortical T cell areas (red). In the spleen, CD8α+ DC (blue; arrows and inset) were identified in the PALS (red), and included those in close proximity to arterioles (original magnification, ×100). C, PCR detection of donor-specific DNA (MHC class II; IAb) in the draining popliteal lymph node (LN). Amplification of β-actin was used as the internal positive control.]

![FIGURE 9. Three-day MLR responses of bulk, popliteal, and inguinal (combined) lymph node cells from C3H recipients of sorted, overnight-cultured B10 CD8α+ (●) or CD8α− (○) liver DC, 7 days after s.c. administration into the flank of one hindlimb. Stimulator cells were bulk splenocytes of the same allogeneic strain (C; B10), or splenocytes syngeneic with the recipient (●; C3H). The results are the mean ± 1 SD from triplicate cultures, and the data are representative of two separate experiments.]

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overnight culture. In addition, and consistent with previous observations on lymphoid tissue DC (11), GM-CSF increased the viability and recovery of both liver DC subsets nonselectively.

The T cell stimulatory capacity of DC is believed to depend in part on their expression of the costimulatory molecules CD80 and CD86, which interact with CD28 on T cells (45). Given the immature phenotype of freshly isolated CD8α+ and CD8α– liver DC, only cells induced to mature by overnight culture were tested for T cell stimulatory activity in MLR. It was not surprising that these overnight-cultured DC exhibited potent allostimulatory ability, which was equivalent on a per cell basis. These observations on the Ag-presenting capacity of CD8α+ DC isolated from the liver are consistent with recent reports of the potent immunostimulatory properties of keyhole limpet hemocyanin- or OVA peptide-pulsed CD8α+ (and CD8α–) DC, isolated from lymphoid tissue and adoptively transferred into normal or OVA-TCR transgenic mice (18, 20). Although in the present study we did not quantify Th cytokines induced in MLR cultures, the latter studies demonstrated the induction of a predominant Th1 cytokine response following administration of Ag-pulsed CD8α+ DC compared with a Th2 or a mixed Th1/Th2 response by CD8α– DC (18–20). Collectively, our observations differ from earlier reports that CD8α+ DC isolated from lymphoid tissues of normal mice were less efficient in the induction of allogeneic T cell proliferation compared with classic MDC. In these earlier studies the reduced ability of CD8α+ DC, compared with that of CD8α– DC, to stimulate the proliferation of CD4+ T cells was ascribed to differential cell surface expression of the death-inducing molecule CD95L (28). In the present investigation we were unable to confirm surface expression of CD95L on either freshly isolated or overnight-cultured CD8α+ or CD8α– DC using mAb staining and flow cytometry. However, RT-PCR analysis demonstrated gene transcripts for CD95L in both freshly isolated and overnight-cultured, sorted CD8α+ and CD8α– liver DC. Thus, it is possible that CD8α+ DC in nonlymphoid tissues, in contrast to those in thymus or spleen, do not express surface CD95L constitutively. Moreover, it has been suggested that CD95L expression by CD8α+ DC may be induced following interaction with naïve T cells (46), a process that usually occurs in the T-dependent regions of lymphoid tissues.

The in vivo significance of our in vitro functional observations was examined by adoptive transfer of overnight-cultured, sorted CD8α+ or CD8α– DC into unmodified, fully allogeneic recipients. The s.c. footpad route of injection was chosen, as this is a conventional approach to the evaluation of DC trafficking and homing in vivo (47). We were able to show for the first time, as has been classically defined for MDC, that mature CD8α+ DC trafficked from the periphery to the T cell areas of allogeneic host lymphoid tissue (PALS) within 48 h. The sensitive techniques used to detect donor CD8α+ DC in the current study probably account for the apparent conflict with a recent report that CD8α– DC may fail to migrate to draining lymph nodes following s.c. injection (48). Ex vivo analysis of anti-donor immune reactivity confirmed the in vivo allostimulatory activity of the overnight-cultured, sorted CD8α+ DC. The allostimulatory capacity of CD8α+ and CD8α– DC was consistent with their surface phenotype (MHC class IIαβ, CD40+, CD80αβ+, and CD86αβ+), and their in vitro activity. These findings in allogeneic recipients are consistent with the adjuvant properties of soluble Ag-pulsed CD8α+ DC observed in syngeneic hosts (18–20).

In conclusion, we have shown for the first time that immature and mature CD8α+ DC populations can be isolated efficiently from mouse liver. Flt3L proved an important in vivo DC growth factor for both subsets and stimulated the production of CD8α+ and CD8α– hepatic DC with equal efficiency. The allostimulatory potential of markedly increased numbers of Flt3L-induced CD8α+ and CD8α– DC in the liver and the trafficking of these cells to T cell areas of secondary lymphoid tissues (31) may account for the acute rejection of mouse liver allografts (usually accepted without immunosuppression) from Flt3L-treated donors (49). Further studies of the functional immunobiology of murine CD8α+ liver DC, particularly freshly isolated, immature cells, may shed further light on the role of these important APC, widely discussed as regulators of immune reactivity (27), in liver inflammation and immunity.

Acknowledgments

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