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Heterogeneity of Dendritic Cells in the Mouse Liver: Identification and Characterization of Four Distinct Populations

Zhe-Xiong Lian,* Tomoyuki Okada,‡ Xiaosong He,* Hiroto Kita,* Yong-Jun Liu,‡ Aftab A. Ansari,§ Kentaro Kikuchi,* Susumu Ikehara,¶ and M. Eric Gershwin**

Liver dendritic cells (DC) are believed to play important roles in liver immunity, autoimmunity, and in the regulation of hepatic allograft acceptance. However, limited information is available on the phenotypes and functions of DC in the liver. To address this issue, we isolated DC from murine liver using procedures that do not involve collagenase and characterized the freshly isolated DC population that had not been subjected to in vitro expansion. Thence, based on the expression of CD4, B220, and CD11b, four subsets or groups of hepatic NK.1.1\(^{-}\)CD11c\(^{+}\) DC were identified with the following phenotypes: B220\(^{+}\)CD4\(^{+}\), B220\(^{+}\)CD4\(^{-}\), B220\(^{-}\)CD11b\(^{+}\), and B220\(^{-}\)CD11b\(^{-}\). Each subset was further characterized both phenotypically and functionally. In addition to unique phenotypic expression, each subset displayed different allostimulation capability in mixed lymphocyte reaction assays. All four groups developed DC morphology following in vitro culture with activation agents and synthesized distinct patterns of cytokines in response to different stimuli. Taken together, our results suggest that groups I and II are IFN-\(\alpha\)-producing plasmacytoid DC, group III cells are myeloid-related DC, while group IV is a heterogeneous population containing both myeloid- and lymphoid-related DC. Our results demonstrate the highly heterogeneous nature of hepatic DC, which is in agreement with the unique requirements for APC in the complex liver environment. The Journal of Immunology, 2003, 170: 2323–2330.

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3 Abbreviations used in this paper: DC, dendritic cell; HMNDC, hepatic mononuclear DC; HMNC, hepatic mononuclear cell; Gr., group; ODN, oligodeoxynucleotide; LN, lymph node; RT, reverse transcriptase; PGN, peptidoglycan; BM, bone marrow; TLR, Toll-like receptor; IPDC, IFN-\(\alpha\)-producing DC; TC, Tri-color; poly(I:C), polyinosinic-polycytidylic acid.

Materials and Methods

Mice

C57BL/6J (H-2\(^{b}\), B6), B6 RAG\(^{-/-}\), and BALB/CJ (H-2\(^{d}\)) mice (8-12 wk-old) were obtained from The Jackson Laboratory (Bar Harbor, ME). They were maintained under pathogen-free conditions and studied at 8–12 wk of age.
Isolation of hepatic mononuclear cells (HMNC)

C57BL/6 mice were anesthetized with methoxyflurane (Metofane; Mallinckrodt, Verona, Madison, WI) in a 50% oxygen-50% nitrous oxide mixture, after their abdomens were opened, a needle was inserted into the portal vein. The inferior vena cava was cut to enable blood outflow and livers were processed with one of the following three procedures: method 1, the liver was perfused with 6 ml of PBS, removed, and gently pressed through nylon mesh; method 2, the liver was perfused with 6 ml of collagenase IV (Sigma-Aldrich, St. Louis, MO), excised, minced, and then diced into small pieces, and digested in collagenase IV solution for 20 min at 37°C (18); the digested tissue was filtered through nylon mesh to remove debris and connective tissue; method 3, the liver was first perfused with PBS as described in method 1, then perfused with 6 ml of liver perfusion medium (Life Technologies, Rockville, MD), followed by an injection of 6 ml of liver digestion medium (Life Technologies). Following the procedures, the liver was removed immediately and gently pressed through nylon mesh. Preliminary experiments demonstrated that <0.01% CD11c+ cells in blood and hence it is unlikely that liver cells are derived from blood. The liver cell suspension, obtained by each of these three methods, was centrifuged at 75 × g for 1 min. The parenchymal cells (pellets) were discarded, while the nonparenchymal cells remaining in the supernatant were collected, washed in PBS, and resuspended in 1.051/g/ml Opti-Prep (Axis-Shield, Oslo, Norway) in complete RPMI 1640 medium. (pellets) were discarded, while the nonparenchymal cells remaining in the supernatant of at least two separate experiments.

Flow cytometry staining and analysis

immunofluorescent labeling was performed as previously described (23). Briefly, 5 to 10 × 10^6 HMNC or NK1.1-depleted HMNC were first incubated with a predetermined optimal amount of anti-CD16/CD32 (except for CD16/32 staining) at 4°C for 5 min to block the FcR, and then stained with various combinations of fluorescence- or biotin-conjugated mAbs. Following washing, Tri-color- (TC) conjugated streptavidin (Caltag Laboratories, Burlingame, CA) was used for the development of the biotin-conjugated Ab-stained cells. FACs data were acquired on a dual laser FACScan (BD Biosciences, San Jose, CA). Similar data was obtained with Coulter Epics XL-MCL (Coulter, Miami, FL). The frequency of cells expressing individual and/or sets of cell surface markers and the mean density of expression of such markers was determined by analyzing a minimum of 50,000 cells after gating out propidium iodide-positive dead cells, and the mean uptake of [3H]thymidine (1 μCi/well) was added during the last 18 h of culture and the cultures harvested using a Skatron (Molecular Devices, Sunnyvale, CA). Proliferation of T cells was measured in a liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA) and the mean uptake of [3H]thymidine deoxyribosyl was determined.

To induce cytokine production, aliquots of 3 × 10^6 HMNC were cultured in vitro with the following stimulating: 10 μg/ml peptidoglycan (PGN) from Staphylococcus aureus (Fluka, Buchs, Switzerland), 10 μg/ml lipoteichoic acid from S. aureus (Sigma-Aldrich), 10 μg/ml LPS from Salmonella minnesota serotype Re595 (Sigma-Aldrich), 50 μg/ml polyinosinic-polycytidylic acid (poly(I:C)) (Sigma-Aldrich), or 2 μM CpG ODN 2216, in complete RPMI 1640 for 48 h. Supernatants were collected and analyzed for the following cytokines, using different ELISA kits: IFN-α (Performance Biomedical Laboratory, New Brunswick, NJ), IL-12 and IL-12 p70 (BioSource International, Camarillo, CA), IL-12 p40, IL-6 and TNF-α (R&D Systems), and IFN-γ (e-Bioscience).

RNA isolation and RT-PCR analysis

Total RNA was isolated from FACS-sorted HMNC of B6 mice using the RNAeasy Mini kit (Qiagen, Santa Clara, CA). To eliminate DNA contamination, the RNA samples were incubated with DNase I (Life Technologies) at room temperature for 15 min. DNase I was then inactivated in the RNA samples by the addition of EDTA and incubation at 65°C for 10 min. The treated RNA samples were used to synthesize first strand cDNA using Superscript II reverse transcriptase (RT; Life Technologies) following the manufacturer’s instructions. The primers used for the RT reaction are listed in Table I. A GeneAmp PCR System 9700 (PerkinElmer/ Applied Biosystems) was used with an initial denaturation step of 94°C for 10 min, followed by 30 cycles (except for β-actin in which case 25 cycles were used) of 94°C for 1 min, 55°C for 1 min 30 s, 72°C for 1 min, and a final elongation step of 72°C for 7 min. PCR products were separated on a 1.5% agarose gel containing ethidium bromide. A 100-bp DNA ladder standard (Life Technologies) was used as a size marker.

Results

Isolation of HMNC without enzyme-perfusion

For each isolation procedure, HMNC were isolated from the liver of 20–40 mice, stained with FITC anti-NK1.1, PE anti-CD11c, TC anti-CD11b, PE-Cy5 anti-CD4, and allophycocyanin anti-CD230. After washing twice, the cells were sorted using a 10-parameter MoFlo cell sorter (Cytometry, Fort Collins, CO) into the following groups (Gr.): Gr. I, NK1.1 CD11c+ B220+ CD4+; Gr. II, NK1.1 CD11c+ B220+ CD4−; Gr. III, NK1.1 CD11c+ B220+ CD11b+; and Gr. IV, NK1.1 CD11c− B220+ CD11b+. The purity of sorted cells, based on the above phenotypic expression of cell surface markers, was always >97%. Aliquots of 2 × 10^5 sorted HMNC were cultured in 200 μl of complete RPMI 1640 medium (Life Technologies) containing 10% FCS, 20 mM HEPES, 2-ME, penicillin, and streptomycin in flat-bottom 96-well plates with or without the following supplements: recombinant mouse IL-4 (25 ng/ml), recombinant mouse GM-CSF (5 ng/ml) (R&D Systems, Minneapolis, MN), and phosphorothiolated CpG-oligodeoxynucleotide (ODN) 2216 (2 μM) (ggG GGA CGA TCG TCG ggg ggg; provided by TriLink BioTechnologies, San Diego, CA).

Detection of apoptotic cells

Sorted HMNC were cultured with or without CpG ODN 2216 (2 μM) for 18 h. Apoptotic cells were detected by annexin V staining using Annexin Apoptosis Detection kits (BD Pharmingen). Briefly, following in vitro culture, HMNC were washed twice and resuspended in binding buffer (10 nM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). The cells were then treated with a predetermined optimal concentration of FITC-conjugated annexin V for 15 min at room temperature in the dark, washed, and then analyzed on a FACSscan flow cytometer (BD Biosciences).

T cell stimulation and cytokine production

HMNC subsets from B6 (H-2b) mice, either freshly isolated or stimulated for 24 h, were gamma-irradiated (28 Gy). CD4+ T cells were isolated from BALB/c (H-2g) mice. Briefly, a lymphocyte suspension derived from a pool of lymph nodes (LN) and spleen was overlaid onto Histopaque-1,077 and centrifuged for 20 min at 750 × g. Low density lymphocytes were collected from the interface, washed with PBS, and resuspended in complete medium. CD4+ T cells were purified from the low density lymphocytes by positive selection with CD4+ MicroBeads and MiniMacs (Miltenyi Biotec). Aliquots of 1 × 10^6 purified CD4+ T cells were cocultured in triplicate with the gamma-irradiated HMNC in 200 μl of complete medium for 4 days in round-bottom 96-well plates. [3H]Thymidine (1 μCi/well) was added during the last 18 h of culture and the cultures harvested using a Skatron (Molecular Devices, Sunnyvale, CA). Proliferation of T cells was measured in a liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA) and the mean uptake of [3H]thymidine deoxyribosyl was determined.

To induce cytokine production, aliquots of 3 × 10^6 HMNC were cultured in vitro with the following stimulating: 10 μg/ml peptidoglycan (PGN) from Staphylococcus aureus (Fluka, Buchs, Switzerland), 10 μg/ml lipoteichoic acid from S. aureus (Sigma-Aldrich), 10 μg/ml LPS from Salmonella minnesota serotype Re595 (Sigma-Aldrich), 50 μg/ml polyinosinic-polycytidylic acid (poly(I:C)) (Sigma-Aldrich), or 2 μM CpG ODN 2216, in complete RPMI 1640 for 48 h. Supernatants were collected and analyzed for the following cytokines, using different ELISA kits: IFN-α (Performance Biomedical Laboratory, New Brunswick, NJ), IL-12 and IL-12 p70 (BioSource International, Camarillo, CA), IL-12 p40, IL-6 and TNF-α (R&D Systems), and IFN-γ (e-Bioscience).
be determined whether the phenotype and functions of HMNDC prepared with these procedures are representative of those naturally present in vivo. To evaluate the impact of different isolation procedures on HMNC, we examined the autofluorescence of unstained HMNC. As shown in Fig. 1, when HMNC were prepared with collagenase IV perfusion (method 2) or digestion medium perfusion (method 3), 4.7–9.2% of the bulk HMNC, as well as CD11c+ cells, were autofluorescent. In contrast, HMNC isolated by the PBS perfusion procedure we developed (method 1) contained <1% of autofluorescent cells. This result indicates that digestion of liver tissue with collagenase increased the autofluorescent cells of isolated HMNC. One to 10^6 of HMNC were isolated per normal mouse liver by method 1, while 1.5 to 2.0 x 10^6 HMNC were isolated using methods 2 or 3. However, there was no significant difference in the absolute number of CD11c+ cells recovered by each of these methods. Therefore, method 1 was used to isolate HMNC for all of the experiments in this study.

Four sublineages of the NK1.1^+ CD11c^+ HMNC population defined by the surface markers B220, CD4, and CD11b

HMNC populations, isolated from mouse livers by method 1, were analyzed for expression of the following cell surface markers: CD11c, NK1.1, CD3, TCRβ, CD19, CD205, CD4, and CD11b, after gating for lymphocytes and monocytes based on their forward scatter vs side scatter profile (data not shown) and exclusion of propidium iodide-positive dead cells by their PE channel fluorescence. Approximately 29% of NK1.1^+ HMNC were CD11c^+ (Fig. 2A), which comprised 19.1% of HMNC. Surprisingly, the majority of NK1.1^+ HMNC also expressed CD11c, although at a lower level (Fig. 2A), and preliminary data from other tissues, including spleen, LN, bone marrow (BM), thymus, and PBMC that NK1.1^+ cells also contain CD11c^+ cells (data not shown). Within the NK1.1^+ population, CD11c^+ HMNC lacked lineage markers for T cells (CD3 and TCRβ), B cells (CD19), and CD205 and were negative for cytoplasmic Ig (data not shown), indicating these are hepatic DC (HMNDC). The expression of B220 (CD45R) on the NK1.1^+ CD11c^+ HMNDC was heterogeneous (Fig. 2B), as indicated by the existence of two subpopulations characterized by B220^-^ and B220^+^ cells, respectively (Fig. 2B). Furthermore, we found that CD4 was expressed by 42% of NK1.1^+ CD11c^+ B220^-^ cells, and that CD11b was expressed by 54% of NK1.1^+ CD11c^-^ B220^-^ cells (Fig. 2C). Taken together, based on the expression of B220, CD4, and CD11b, we were able to define the following four distinct subsets within the NK1.1^+ CD11c^+ HMNDC population: Gr. I, NK1.1^+ CD11c^-^ B220^-^ CD4^-^; Gr. II, NK1.1^+ CD11c^-^ B220^-^ CD4^+^; Gr. III, NK1.1^+ CD11c^-^ B220^-^ CD11b^-^; and Gr. IV, NK1.1^+ CD11c^-^ B220^-^ CD11b^-. Similar groups of HMNDC were isolated from the liver of B6/RAG^-/-^ mice (Fig. 2E) and nonobese diabetic/SCID mice (data not shown) that were deficient in T and B cells, indicating that the HMNDC groups identified with these markers are not B cells.

Next, we used RT-PCR analysis to examine the subset-specific expression of several markers in the four subsets of HMNDC defined by Ab staining for cell surface markers. First, because all of the Gr. I cells and a fraction of Gr. IV cells stained positive for the cell surface CD4 molecules (Fig. 2C), we examined the CD4 mRNA transcript in the four subsets to rule out the possibility that the detection of the cell surface CD4 molecule was due to passive Ag uptake from T cells (24). As shown in Fig. 2D, CD4 mRNA was readily detected in Gr. I and, at a lower level, in Gr. IV, but not in cells from the other two groups. Similar results were obtained in the same subsets of HMNDC isolated from RAG^-/-^/ mice (data not shown). This indicates that the CD4 Ag was synthesized de novo in the Gr. I cells and possibly in some of the Gr. IV cells.

Second, as pan-B cell marker B220 was detected on Gr. I and Gr. II HMNC, we examined mRNA transcripts for the following B cell-related marker genes: the B lineage commitment indicator μα (25–27), the TdT gene that mediates addition of nongermline-encoded nucleotides to V-D and D-J junctions of Ig H chain genes (28), and a B-restricted component of the pre-B cell receptor complex, mb-1 (Igα) and B220 (Igβ) (26, 29, 30). As shown in Fig. 2D, μα, TdT, mb-1, and B220 were detected only in Gr. I and Gr. II HMNC, confirming that these two subsets are related to B cells.

Finally, as Rel B has been reported to be an important marker for myeloid DC (31), we examined the mRNA transcript for this marker in the four HMNDC subsets. Fig. 2D shows that Rel B was expressed in Gr. III and IV, but not in Gr. I and II, cells (Fig. 2D). Taken together, these results suggest that Gr. I and II are related to plasmacytoid DC, while Gr. III and IV are related to myeloid and lymphoid DC.

We examined the morphology of the four subsets of FACS-sorted HMNDC after May-Giemsa staining (Fig. 3A). Freshly isolated Gr. I and II cells showed a homogeneous plasmacytoid morphology that was characterized by a round shape, smooth surface, an oval or indented nucleus, and a prominent perinuclear pale

<table>
<thead>
<tr>
<th>Table I. PCR primer sequences</th>
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<tbody>
<tr>
<td><strong>RT-PCR Primers</strong></td>
</tr>
<tr>
<td><strong>Forward primers</strong></td>
</tr>
<tr>
<td>Rel B</td>
</tr>
<tr>
<td>μα</td>
</tr>
<tr>
<td>TdT</td>
</tr>
<tr>
<td>mb-1</td>
</tr>
<tr>
<td>B220 (Igβ)</td>
</tr>
<tr>
<td>CD4</td>
</tr>
<tr>
<td>β-Actin</td>
</tr>
</tbody>
</table>

FIGURE 1. Autofluorescence of HMNCs prepared by three different methods. Unstained HMNC populations isolated using three different methods (see Materials and Methods) were directly analyzed for fluorescence in the FITC channel and PE channel of the FACScan flow cytometer.
zone. Group IV cells possessed a round shape, smooth surface and irregular or hyperlobulated nucleus. In contrast to the other three groups, the morphology of Gr. III cells were heterogeneous, with some cells possessing a more abundant cytoplasm, dendrites, and an irregular or hyperlobulated nuclei.

Surface phenotype of HMNDC

A more extensive cell surface phenotype analysis of the four HMNDC subsets was conducted using a panel of 14 Abs and a FACS analysis, either using freshly isolated cells or after a 24-h in vitro culture for maturation of DC. A freshly isolated pool of the HMNDC population was first depleted of NK1.1+ cells with magnetic beads as described in Materials and Methods, then stained with Abs against MHC class I, MHC class II, CD80, CD86, CD40, and CD45RA (Fig. 4, Table II, and data not shown). The vast majority (>95%) of all the four HMNDC subsets expressed high levels of MHC class I, only a minor proportion (<5%) of Gr. III and IV HMNDC lacked this marker. All Gr. I and >95% of Gr. II HMNDC expressed low levels of MHC class II, and a minor proportion (<5%) of Gr. II HMNDCs lacked this marker, whereas ~50% of Gr. III and 35% of Gr. IV HMNDC expressed high levels of this marker. Gr. I and Gr. II cells were negative for the T cell costimulatory molecules CD40, CD80, and CD86 as well as the cytokine receptors CD123 and CD127. In contrast, these markers were expressed by Gr. III and Gr. IV cells. In addition, while most of the HMNDC were CD24+, a minor proportion of a small number of CD24low cells were consistently observed in Gr. I and Gr. II but not in Gr. III and Gr. IV. Although almost 97% of Gr. I cells (>90%) expressed relatively high levels of Ly6C (Ly6C++), ~29% of Gr. II cells did not express this marker at a lower level (Ly6C+). In contrast, 93% of Gr. III and 97% of Gr. IV cells were negative for Ly6C++ and most Gr. IV cells lacked this marker. In addition, both Gr. I and Gr. II expressed high levels of CD45RA and both Gr. III and Gr. IV lack this marker (data not shown).

After 24 h in culture with CpG ODN 2216 (for Gr. I and Gr. II cells) or IL-4/GM-CSF (for Gr. III and Gr. IV cells), all four populations of HMNDC matured and their expression levels of MHC class II, CD40, CD80, and CD86 were up-regulated (Fig. 4B).

Promotion of HMNDC survival

We examined the effect of CpG ODN 2216 on the development of HMNDC by in vitro culturing of the four FACS-sorted HMNDC subsets with or without this oligonucleotide for 18 h (32, 33). When cultured without the oligonucleotide, all four subsets of HMNDC showed little or no viability (<10%), as indicated by >90% of cells undergoing apoptosis (Fig. 5). With the addition of CpG ODN 2216, the frequency of surviving cells increased (Fig. 5). Gr. IV cells survived with a viability of >70%, while >50% of Gr. III cells survived. Survival of Gr. I and Gr. II DC cells was also increased but to lower levels compared with Gr. III and Gr. IV.

After a 24-hour culture in the presence of CpG ODN 2216, the surviving cells in each group matured as indicated by development
of numerous dendritic processes and a clear DC morphology. The two left panels of Fig. 3B reflect the morphology of mature DC derived from Gr. I and Gr. II. When cultured with IL-4 and GM-CSF, almost all Gr. III and Gr. IV cells developed dendritic processes and displayed a clear DC morphology (Fig. 3B, two right panels). In contrast, Gr. I and Gr. II cells did not survive under this culture condition (data not shown).

Alloactivation capability of the HMNDC subsets

An important defining characteristic for DC is their ability to induce potent alloactivation. The four subsets of HMNDC were thus examined for their capacity to stimulate allogeneic T cell proliferation by a mixed leukocyte reaction. Freshly sorted subsets of HMNDC from B6 mice were gamma-irradiated and mixed with allogeneic CD4+ T cells from BALB/c mice at different ratios and cultured for 4 days. As shown in Fig. 6A, both Gr. III and Gr. IV cells were equally potent stimulators for inducing proliferation of allogeneic CD4+ T cells. In contrast, Gr. I and Gr. II cells failed to induce detectable proliferation of allogeneic CD4+ T cells. After 24 h of in vitro culture in the presence of CpG ODN 2216 (Gr. I and Gr. II) or IL-4 + GM-CSF (Gr. III and Gr. IV) to induce maturation of HMNDC, the capacity of allogeneic stimulation increased dramatically in Gr. III and Gr. IV and moderately in Gr. II, but not in Gr. I (Fig. 6B). The result is in agreement with the

Table II. Comparison of GR. I, GR. II, GR. III, and GR. IV HMNDCs

<table>
<thead>
<tr>
<th>Ag</th>
<th>Gr. I CD11b B220+ CD4+</th>
<th>Gr. II CD11c B220+ CD4+</th>
<th>Gr. III CD11c B220+ CD11b+</th>
<th>Gr. IV CD11c B220+ CD11b+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–/+</td>
</tr>
<tr>
<td>CD8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD11b</td>
<td>–</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
</tr>
<tr>
<td>CD16/32</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD40</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>CD80</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>CD86</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Ly6C</td>
<td>++++</td>
<td>++</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>mRNA gene expression</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD4</td>
<td>1.34</td>
<td>0.95</td>
<td>0.85</td>
<td>–</td>
</tr>
<tr>
<td>μc</td>
<td>0.95</td>
<td>1.00</td>
<td>1.00</td>
<td>–</td>
</tr>
<tr>
<td>mb-1 (Igα)</td>
<td>0.77</td>
<td>0.81</td>
<td>0.81</td>
<td>–</td>
</tr>
<tr>
<td>B29 (Igβ)</td>
<td>0.44</td>
<td>0.27</td>
<td>0.27</td>
<td>–</td>
</tr>
<tr>
<td>Rel B</td>
<td>–</td>
<td>–</td>
<td>0.95</td>
<td>0.30</td>
</tr>
<tr>
<td>Immunogenic stimulation</td>
<td>2,606 ± 776</td>
<td>6,639 ± 2,281</td>
<td>71,015 ± 10,282</td>
<td>61,247 ± 7,233</td>
</tr>
</tbody>
</table>

a Expression ranges: – (<5%), +/- (5–30%), + (31–60%), ++ (61–80%), and ++++ (80%+).
b Ratio of the intensity of the gene band compared with the corresponding β-actin band using NIH Image software.
c Mean ± SEM, 1 × 105 CD4 T cells: 1 × 104 HMNDCs.
different extents of survival of the four HMNDC subsets in culture (Fig. 5).

Production of cytokines by HMNDC subsets stimulated with Toll-like receptor (TLR) ligands

Ten members have been identified in the mammalian TLR family (34–37) which are thought to recognize different ligands derived from bacteria and viruses. To test the hypothesis that the four HMNDC subsets respond differentially to various TLR ligands, we stimulated sorted HMNDC with four TLR ligands and examined the production of cytokines IFN-α, IL-12, IL-6, and TNF-α. As shown in Fig. 7, the TLR9 ligand CpG ODN 2216 was the only stimulus that induced IFN-α, which was produced at high levels by Gr. I and Gr. II cells and at a low level by Gr. IV cells. In addition, CpG ODN 2216 triggered production of large amounts of IL-12 (p40), IL-6, and TNF-α from Gr. III and Gr. IV, as well as lower levels of TNF-α by Gr. I and Gr. II. No IFN-γ was produced by any group stimulated with the oligonucleotide. Small amounts of IL-12 (p40) were detected from Gr. III and Gr. IV HMNDC stimulated by the TLR2 ligand PGN, TLR3 ligand poly(I:C), and TLR4 ligand LPS. Preinflammatory cytokines IL-6 and, especially, TNF-α were predominantly produced by Gr. III cells stimulated with the TLR2 ligand PGN, TLR3 ligand poly(I:C), and TLR4 ligand LPS. These results confirm that the four HMNDC groups are functionally distinct populations.

Discussion

There is considerable interest in defining the precise sublineage of DC and functions (6). The establishment of such criteria gains importance because presentation of Ag by DC sublineages can lead to a wide variety of immune responses ranging from activation of Th cells to the induction of Ag-specific tolerance and regulation of immune responses (14, 15). Such detailed studies have led to the observation that DC subsets can be distinguished by their characteristic localization, requirements for growth factors, and response to activating stimuli (5, 6). To date, a total of five DC subtypes have been found in lymphoid tissues (38), three of which include types of DC localized within mouse spleen and the other two within the thymus (24).

The liver is the largest organ in the body with crucial metabolic activities. It is also a primary target for autoimmune diseases and infections (39). In the liver, large amounts of oxygenated blood carried by the hepatic artery are used to mobilize energy from dietary components. Toxic compounds, accidentally ingested along with food, are transported from the bowel via the portal vein and detoxified (40). Therefore, the liver has significant requirements for unique defense mechanisms to protect itself from infections, toxins, and malignant cells as well as from unwarranted responses to harmless dietary proteins. To meet these heterogeneous requirements, the liver hosts various unique resident cell types with immune potential, a lymphoid population, and cells with accessory immunologic functions such as DC, Kupffer cells, and biliary epithelial cells (39). The repertoire of lymphocytes present in the liver differs dramatically from those in the other parts of the body, and may have a specialized role in the immune response (41). Such cells may have unique characteristics to serve as APC. Therefore, identification and characterization of APC in the liver is highly relevant to understanding immune cells and their functions unique to the liver.

In mice, CD11c has been used widely as the primary marker for DC, which is expressed by DC isolated from a variety of lymphoid...
and nonlymphoid tissues (42). To our surprise, a large fraction of HMNC that express NK1.1, a marker for NK (43, 44) and NKT cells (26, 50), also express CD11c, although at a lower level. The implication of the CD11c⁻NK1.1⁺ phenotype is unknown at this time, although it may suggest that NK and DC share a common developmental pathway (46). Of note, it has been suggested that BM B220⁺CD19⁻ cells also contain the NK cell progenitor (47).

The developmental potential of these cells needs to be further analyzed. Because murine liver DC have been shown to potently activate liver NK cells to release the proinflammatory cytokines IFN-γ and IL-4 (22), it is important to exclude the CD11c⁻NK1.1⁺ cells from the liver DC isolates to avoid contamination with NK or NKT cells, which may confound the phenotypic and functional analyses for DC.

In the current work, we have identified four subsets of HMNC with distinct cell surface markers and functional phenotypes. Selected characteristics of these subsets are summarized in Table II. Among the four groups of HMNC, Gr. I and Gr. II displayed several similarities to human plasmacytoid DC and murine LN and spleen IFN-α-producing DC (IPDC) (32, 33, 48). These include their plasmacytoid morphology (32, 33, 48), immature phenotype when freshly isolated (33), rapid apoptosis (33), lack of Ag-processing capacity (32), and production of IFN-α after CpG ODN stimulation (49). These similarities strongly suggest that the Gr. I and Gr. II HMNDC we described herein are the murine liver IFN-α-producing DC (IPDC) (32, 33, 48). Among the four groups of HMNDC, Gr. I and Gr. II displayed a potent allogeneic T cell activation capacity by the mixed leukocyte reaction assay. Gr. III cells express high levels of surface CD11b and Rel B mRNA (31), suggesting that they are myeloid-related DC. Gr. IV is a heterogeneous population that contains a fraction of CD4⁺ as well as a fraction of CD8⁺ cells. This population synthesizes a lower but still significant level of IFN-α compared to Gr. I and Gr. II cells. Therefore, this group may contain both CD11b⁺ myeloid progenitors and CD4⁺ or CD8⁺ lymphoid DC progenitors.

It is thought that different types of DC express distinct sets of TLRs, which are essential for the recognition of pathogen-associated molecular patterns by the innate immune system and for the mediation of specific immune responses to various pathogens. For example, some antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway (53). TLR9 is thought to be essential for the recognition of bacterial DNA sequences (CpG-DNA) (35). TLR2 and TLR4 recognize PGN, lipopeptides, and LPS (53), while TLR3 is associated with specific response to poly(I:C) (53). We demonstrate herein that the four groups of HMNDC produced different cytokines when stimulated with various TLR ligands. Gr. I and Gr. II cells are potent producers of IFN-α, a strong inhibitor of viruses and other intracellular pathogens, while Gr. III and Gr. IV cells produced high levels of IL-12, an important regulator for differentiation of type 1 Th cell responses (5). Gr. III is the most potent producer of proinflammatory cytokines IFN-6 and TNF-α. These results suggest that liver HMNDC subsets play distinct roles in both innate and adaptive immune responses.

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References
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