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Human Dendritic Cell Subsets from Spleen and Blood Are Similar in Phenotype and Function but Modified by Donor Health Status

Diana Mittag,*1 Anna I. Proietto,*1 Thomas Loudovaris,‡ Stuart I. Mannering,‡ David Vremec,† Ken Shortman,† Li Wu,†,2 and Leonard C. Harrison*,2

Mouse dendritic cells (DC) have been extensively studied in various tissues, especially spleen, and they comprise subsets with distinct developmental origins, surface phenotypes, and functions. Considerably less is known about human DC due to their rarity in blood and inaccessibility of other human tissues. The study of DC in human blood has revealed four subsets distinct in phenotype and function. In this study, we describe four equivalent DC subsets in human spleen obtained from deceased organ donors. We identify three conventional DC subsets characterized by surface expression of CD1b/c, CD141, and CD16, and one plasmacytoid DC subset characterized by CD304 expression. Human DC subsets in spleen were very similar to those in human blood with respect to surface phenotype, TLR and transcription factor expression, capacity to stimulate T cells, cytokine secretion, and cross-presentation of exogenous Ag. However, organ donor health status, in particular treatment with corticosteroid methylprednisolone and brain death, may affect DC phenotype and function. DC T cell stimulatory capacity was reduced but DC were qualitatively unchanged in methylprednisolone-treated deceased organ donor spleen compared with healthy donor blood. Overall, our findings indicate that human blood DC closely resemble human spleen DC. Furthermore, we confirm parallels between human and mouse DC subsets in phenotype and function, but also identify differences in transcription factor and TLR expression as well as functional properties. In particular, the hallmark functions of mouse CD8α+ DC subsets, that is, IL-12p70 secretion and cross-presentation, are not confined to the equivalent human CD141+ DC but are shared by CD1b/c+ and CD16+ DC subsets. The Journal of Immunology, 2011, 186: 6207–6217.

Dendritic cells (DC) process and present self and foreign Ags to T cells and are regulators of the adaptive immune response. DC comprise a network of subsets that are phenotypically, functionally, and developmentally distinct (1). Understanding this diversity is important in targeting DC for immunoregulatory therapies. DC subsets identified in mouse lymphoid organs are broadly classified into two groups: conventional DC (cDC) and plasmacytoid DC (pDC). cDC include both tissue-resident and migratory subsets. In mouse spleen, two major resident cDC subsets, CD8α+CD11b+ (CD8α+) and CD8α+CD11b− (CD8α−) cDC, have been identified (2). CD8α+ cDC are located within the marginal zones of the spleen (3), migrate to the T cell areas in response to TLR stimuli (3, 4), strongly activate CD4+ T cells, and produce inflammatory chemokines (5). In contrast, CD8α− cDC have a unique ability to cross-present Ag to CD8+ T cells to generate CTL responses, as well as being the major producers of the Th1-polarizing cytokine IL-12p70 (6). pDC are present in the spleen and other lymphoid tissues but also circulate within the blood. They mature following TLR stimulation and have a weak capacity to stimulate T cells but are the major producers of type I IFNs (IFN-α) (7–10) required for pathogen clearance.

Identification and functional analysis of cDC and pDC in human spleen are of great interest given vaccine strategies that target DC in mouse spleen can boost immune responses (11). However, the relationship between mouse and human DC subsets has until recently remained elusive, making translation difficult. Moreover, studies of human DC, including monocyte-derived DC (12), have been largely restricted to blood due to the limited availability of human spleen tissue.

Human blood cDC are identified as lineage−CD11c+HLA-DR+, however, CD8α, the major marker used to segregate mouse cDC subsets, is not expressed by human cDC (13). Instead, CD1b/c (BDCA1), CD141 (BDCA3), and CD16 are used to distinguish three human cDC subsets. Blood pDC are CD11c+CD123+ (IL-3R+) and CD304+ (BDCA4+) (14, 15) and secrete IFN-α in response to TLR stimulation. Activated cDC produce proinflammatory cytokines (16), similar to their mouse counterparts. Genome-wide expression profiling has been used to assign mouse DC to the three human cDC counterparts (17). CD141+ human cDC were suggested to be the equivalent to mouse CD8α+ cDC,
supported by other similarities: CD141+ cDC also selectively express NECL2 and CLEC9A (18–21). Functional analysis of human blood DC subsets is limited by low cell yields. However, recently, four studies demonstrated the capacity of human blood CD141+ DC to cross-present and to produce IL-12p70, two functions in which mouse spleen CD8α+ cDC excel (22–25). CD16+CD11c+HLA-DR+ cells have been described as a subset of both DC (14) and monocytes (26) because they have properties of both cell types, and they are termed proinflammatory monocytes because they produce large amounts of TNF-α and little IL-10 (27). The CD11c+ human cDC subset may be the equivalent of the CD8α+ DC. However, the genetic evidence is not as strong for the cDC subsets as for the pDC (17), and functional data are lacking. Most studies have focused on studying CD141+ cDC, the putative equivalent of the CD8α+ DC. A comprehensive functional comparison of all three human cDC subsets has not been reported.

Mouse spleen resident cDC are thought to develop locally from blood precursors (28). It is not clear whether cDC in human blood are precursors or similar to fully differentiated, functional human spleen DC subsets. Initial histology-based studies of human spleen revealed heterogeneity of DC (21, 29), but detailed phenotypic and functional studies of human spleen DC are lacking, due to restricted access to human spleen of suitable quality. Moreover, the health status of organ donors needs to be considered in studies of DC.

To our knowledge, this is the first study characterizing phenotype and function of human spleen DC subsets. We used deceased organ donors in which life-support was maintained prior to brain death, resulting in optimal quality of the tissue (30). We developed new methods to isolate and functionally characterize DC subsets from spleen for a comprehensive comparison of all human spleen and blood DC subsets, and addressed organ donor-related effects on DC.

Materials and Methods

*Human blood and spleen donors*

Cells were isolated from human blood buffy coats (Australian Red Cross Blood Service, Melbourne, VIC, Australia) or blood of healthy volunteers with informed consent. Human spleen tissue was obtained from deceased organ donors (heart beating, brain dead, or after cardiac death) with informed consent from next of kin. The spleen was perfused with 4 1 Ross solution (Orion Laboratories, Balcatta, WA, Australia) and 2 1 UW solution (31) to remove circulating blood. Organ donors were routinely given i.v. solution (Orion Laboratories, Balcatta, WA, Australia) and 2 l UW solution (31) to remove circulating blood. Organ donors were given i.v. the glucocorticosteroid, methylprednisolone (MP), and antibiotics. PerCP-Cy5.5 streptavidin (BD Biosciences) was used as a secondary Ab.

*Isolation of blood and spleen cells*

Spleen tissue was disrupted with scissors and then digested with collagenase (0.5 mg/ml; Roche, Mannheim, Germany) at 3 g tissue/20 ml RPMI (2 mg/ml; Worthington Biochemical, Lakewood, NJ) in the presence of DNase (0.5 mg/ml; Roche, Mannheim, Germany) and 2 l UW solution (31) to remove circulating blood. Organ donors were routinely given i.v. the glucocorticosteroid, methylprednisolone (MP), and antibiotics. PerCP-Cy5.5 streptavidin (BD Biosciences) was used as a secondary Ab.

Flow cytometry and cell sorting of DC

Fc receptors were blocked using mouse Ig (Jackson Immunoresearch Laboratories, West Grove, PA) and then the cells were labeled with Abs against surface markers by resuspending 1 × 106 cells in 10 μl staining solution (human toxicity PBS/FCS-EDTA-containing conjugated mAbs for 25 min and washed with human toxicity PBS/FCS-EDTA. Each mAb was conjugated to R-PE, Quantum Dot 655, or FITC (all Molecular Probes/Invitrogen). Flow cytometry analysis and sorting were performed on the LSRII and FACSAria (BD Biosciences) instruments. Cells were stained with a mixture of FITC- or PE-conjugated Abs against the lineage markers CD3, CD14, CD19, and CD56 (BD Biosciences) to exclude lineage+ cells, and anti–HLA-DR Qdot 655 (clone 2.06), CD11c-PE-Cy7 (BioLegend, San Diego, CA), BDC1-allophycoerythrin or BDC3-allophycoerythrin, BDC4-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), CD11b-PE (BD Biosciences), SIRPa-biotin (Bio-Legend), CD11b-PE (OM1), and CLEC9A-FITC (4C6). PerCP-Cy5.5 streptavidin (BD Biosciences) was used as a secondary Ab.

DC stimulation for analysis of cytokine production and surface activation marker expression

Sorted human blood and spleen DC subsets were cultured at 1 × 106/ml in 96-well round-bottom plates for 24 h with either the natural TLR9 agonists CpG A and CpG C (1 μm) (Geneworks, Hindmarsh, SA, Australia), soluble trimeric CD40L (1 μg/ml) (gift from Dr. D. Lynch, Agen, Thousand Oaks, CA), human GM-CSF (100 ng/ml) (eBioscience, San Diego, CA), human IL-4 (40 ng/ml) (eBioscience), human IFN-γ (40 ng/ml) (Pepro-Tech, Rocky Hill, NJ), and, where indicated, with influenza virus protein preparation (10 μg/ml) (New Caledonia 20/1999 H1N1, World Health Organization [WHO]) and human IFN-α (1000 U/ml) (Roferon-A; Hoffmann-La Roche, Basel, Switzerland). Culture supernatants were collected and stored at −20°C until cytokines were assayed in a 14-plex Milliplex human cytokine bead array (Millipore, Billerica, MA) as per the manufacturer’s instructions.

Allogeneic MLR

Blood CD4+ T cells were isolated using a magnetic bead-based CD4+ T cell-negative isolation kit following the manufacturer’s instructions (Miltenyi Biotec). CD4+ T cells were incubated for 10 min at 37°C with CFSE (0.5 μM) and then washed twice with HBSS. CFSE-labeled purified allogeneic CD4+ T cells (5 × 105 cells/well) from blood were cocultured with sorted blood or spleen DC in a 1:2 se-rial dilution starting from 3 × 106 cells/well, in duplicate for 96 h in 96-well V-bottom plates. T cell proliferation was determined by resuspending cells in human toxicity PBS/5% FCS containing a known number of latex beads (BD Biosciences). Numbers of CFSElo live dividing T cells were determined relative to the number of latex beads added per well.

RNA isolation and quantitative RT-PCR

RNA was prepared from purified DC populations using the RNeasy Mini kit (Qiagen) as per the manufacturer’s instructions. RNA (up to 2 μg) was treated with RNase-free DNase (Promega, Madison, WI) and then reverse transcribed into cDNA using random primers (Promega) and SuperScript II reverse transcriptase (Life Technologies-BRL/Invitrogen). RT-PCR was performed to de- termine the expression of Gapdh, TLR-3, TLR-4, TLR-7, TLR-9, IRF-4, IRF-8, and iκBα using the QuantiTec SYBR Green PCR kit (Qiagen) and a Light Cycler (Hoffmann-La Roche) as per the manufacturers’ instructions. The specific primers for RT-PCR were: Gapdh, 5'-TTCCCTGACACGGCTGC- TTTT-3', 5'-ATTCGCTCCTGCGGAAATGGTCG-3'; TLR3, 5'-GGGCC- CTCCTGAACCTTTT-3', 5'-AAATGTCTCGACCATCTCCT-3'; TLR4, 5'-GTTGCCAACACTCCCTCAGGGTC-3', 5'-GTTGACATCTCCATCAGTTGTC-3'; 5'-TAAT TGGTGGGAGTGGTGTACCTG-3'; TLR9, 5'-GAATTTCCAGGCTGACTGATG- GAGA-3', 5'-ATCGATGGGACAGGAAGAAGCT-3'; IRF4, 5'-TCC AGGTTACCTCTAGTTTCA-3', 5'-CTGTTAGTGCTGATTGTTCT-3'; 5'-TTAG TGGTACACCTTGGATGGTGTACCTG-3'; TLR7, 5'-CTGTTAGTGCTGATTGTTCT-3'; 5'-TAAT TGGTGGGAGTGGTGTACCTG-3'; TLR8, 5'-TTCCCTGACACGGCTGC- TTTT-3', 5'-ATTCGCTCCTGCGGAAATGGTCG-3'; 5'-CTGG TACACCCTCCCTCAGGGTC-3', 5'-GTTGACATCTCCATCAGTTGTC-3'.
Cross-presentation assay

DC subsets were isolated from thawed human blood and spleen cells as described above and seeded at 2000 cells per well in a 96-well U-bottom plate in 150 µl complete medium (human tumor RPMI 1640, 10 mM HEPES, 2 mM Glutamax, 10 mM nonessential amino acids [Invitrogen], 5% pooled human serum [Australian Red Cross Blood Service], 50 µM 2-ME [Sigma-Aldrich]). DC subsets were preincubated with 10 µg/ml influenza virus Ag (New Caledonia 20/1999 H1N1, WHO; the virus had been propagated in the allantoic cavity of embryonated hen’s eggs, inactivated with UV light and formaldehyde, purified by centrifugation, and disrupted with sodium deoxycholate) in the presence or absence of polyinosinic-polycytidylic acid [poly (I:C)] (12.5 ug/ml) or for 24 h at 95˚C was followed by 35–45 cycles of 15 s at 94˚C (denaturation), 20–30 s at 50–60˚C (annealing), and 10–12 s at 72˚C (extension), followed by melting point analysis. The expression level for each gene was determined using a standard curve prepared from 10^{-3} to 10^{-7} pg specific DNA fragment and then expressed as a ratio to Gapdh.

DC exposure to MP in vitro

Blood DC (50,000/well) enriched from PBMC of healthy volunteers were preincubated with influenza Ag (10 µg/ml, inactivated virus, New Caledonia 20/1999 H1N1, WHO) in the presence of MP (100 µg/ml) (Pfizer, West Ryde, NSW, Australia) or solvent for 2.5 h. After washing, the capacity of DC to stimulate T cells (50,000 cells/well) was assessed as CD4+ T cell proliferation (CFSE dilution) and CD8+ T cell clone IFN-γ secretion (ELISPOT), as described above.

Statistics

The p values were determined by the two-tailed paired Student t test for normalized paired values. A p value <0.05 was considered significant. Analyses were performed with GraphPad Prism software v.5 (GraphPad Software, San Diego, CA).

Results

Four DC subsets have been identified in human spleen that resemble those found in human blood

Using the newly established DC isolation protocols for human blood and spleen, we compared DC in blood from healthy living donors with blood and spleen from heart-beating, brain-dead organ donors, as well as spleen from donors after cardiac death (Table I). DC were enriched from PBMC from healthy and organ donor blood and stained for lineage markers, HLA-DR, CD11c, and DC subset-specific markers CD11b/c, CD141, CD16, and CD304, and analyzed by flow cytometry. Within the lineage HLA-DRCD11c+ population we observed CD11b/c+, CD141+, and CD16+ cDC and a subset of HLA-DR1CD11c–CD304+ pDC in both healthy and brain-dead donor blood (Fig. 1A, 1B). However, the frequency of CD16+ DC was reduced in organ donor blood.

Spleens were perfused to remove blood, cut, and digested with collagenase. DC were enriched from splenocytes and stained as described above for PBMC. All four DC subsets present in human blood were also detected in human spleen from deceased organ donors (Fig. 1C, 1D). In 11 tested brain-dead organ donor spleens, the proportions of CD11b/c+, CD141+ cDC, and CD304+ pDC were similar to healthy donor blood (Supplemental Fig. 2), with the exception being CD16+ DC, where the proportion was reduced.

Table I. Characteristics of spleen donors and figures that display experimental data from their tissues

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cause of Death</th>
<th>Known Medical History</th>
<th>Age (y)</th>
<th>Sex</th>
<th>MP Death (h:mm)</th>
<th>BMI</th>
<th>CIT (h:mm)</th>
<th>WIT (h:mm)</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hypoxia</td>
<td>History of hypertension (20 y) and heart disease, chest pain, hypertension medication</td>
<td>45</td>
<td>F</td>
<td>Y</td>
<td>BD</td>
<td>8:30</td>
<td>37.6</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Hypoxia</td>
<td>Hypertension, SOB, heavy drinker</td>
<td>59</td>
<td>M</td>
<td>N</td>
<td>DCD</td>
<td>NK</td>
<td>38.1</td>
<td>0:33</td>
</tr>
<tr>
<td>3</td>
<td>SAH</td>
<td>Kidney stones</td>
<td>57</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>5:38</td>
<td>28.3</td>
<td>2, 3, 4, 5, 6, S2</td>
</tr>
<tr>
<td>4</td>
<td>Myocardial infarction</td>
<td>None</td>
<td>51</td>
<td>F</td>
<td>Y</td>
<td>BD</td>
<td>3:20</td>
<td>28.3</td>
<td>2, 5, 6</td>
</tr>
<tr>
<td>5</td>
<td>Gunshot wound to head (suicide)</td>
<td>Epilepsy since 1995 after gunshot wound (suicide attempt)</td>
<td>57</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>3:33</td>
<td>26.6</td>
<td>1, 5, 6, S2</td>
</tr>
<tr>
<td>6</td>
<td>SAH</td>
<td>Gout</td>
<td>68</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>4:20</td>
<td>24.7</td>
<td>5, 7, S2</td>
</tr>
<tr>
<td>7</td>
<td>ICH</td>
<td>Hemochromatosis, type 2 diabetes</td>
<td>57</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>3:58</td>
<td>25.8</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>SAH</td>
<td>History of hypertension</td>
<td>51</td>
<td>F</td>
<td>Y</td>
<td>BD</td>
<td>10:19</td>
<td>27.7</td>
<td>7, S2</td>
</tr>
<tr>
<td>9</td>
<td>ICH</td>
<td>Arthritis, shingles 3 mo before death, allergy</td>
<td>66</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>0:00</td>
<td>26.9</td>
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</tr>
<tr>
<td>10</td>
<td>Bilateral MCA infarcts</td>
<td>Chronic hip arthritis (but had hip surgery)</td>
<td>71</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>9:37</td>
<td>29.7</td>
<td>7, S2</td>
</tr>
<tr>
<td>11</td>
<td>Suicide by insulin overdose</td>
<td>Type 1 diabetes</td>
<td>42</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>6:01</td>
<td>20.4</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>SAH</td>
<td>Hypertension</td>
<td>56</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>13:35</td>
<td>28</td>
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<td>M</td>
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<td>8:31</td>
<td>29.4</td>
<td>S2</td>
</tr>
<tr>
<td>14</td>
<td>SAH</td>
<td>Type 2 diabetes</td>
<td>57</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>8:27</td>
<td>25.4</td>
<td>S2</td>
</tr>
<tr>
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<td>47</td>
<td>F</td>
<td>Y</td>
<td>BD</td>
<td>4:30</td>
<td>23.6</td>
<td>2, S2</td>
</tr>
<tr>
<td>16</td>
<td>Sepsis</td>
<td>Asthma, rye grass allergy</td>
<td>59</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>NK</td>
<td>37</td>
<td>S2</td>
</tr>
<tr>
<td>17</td>
<td>Gunshot wound brain damage (suicide)</td>
<td>Hepatitis B surface Ab-positive</td>
<td>69</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>6:55</td>
<td>31.1</td>
<td>3, 4</td>
</tr>
<tr>
<td>18</td>
<td>ICH after head trauma</td>
<td>None</td>
<td>17</td>
<td>M</td>
<td>Y</td>
<td>BD</td>
<td>NK</td>
<td>20.1</td>
<td>1, S4</td>
</tr>
<tr>
<td>19</td>
<td>SAH</td>
<td>Heart disease</td>
<td>46</td>
<td>F</td>
<td>N</td>
<td>DCD</td>
<td>NK</td>
<td>33.3</td>
<td>0:24</td>
</tr>
</tbody>
</table>

BD, brain death; BMI, body mass index; CIT, cold ischemia time; DCD, donation after cardiac death; ICH, intracranial hemorrhage; MCA, middle cerebral artery; MP, methylprednisolone; NK, not known; S (for figures), Supplemental; SAH, subarachnoid hemorrhage; SOB, shortness of breath; WIT, warm ischemia time.
Human spleen tissue from donation after brain death is the best available option for studying human spleen DC subsets. It was necessary to address the potential effects of donor health status on DC. Organ donors have been exposed to a variety of stresses and treatments that could cause changes in the DC phenotype. Organs for transplantation are normally obtained from heart-beating donors after brain death, which is accompanied by the release of inflammatory cytokines and chemokines (36). Donations...
after cardiac death occur less commonly. All brain-dead donors but only a few cardiac death donors are treated with the corticosteroid MP.

Although all four DC subsets were present in deceased organ donor blood and spleen, we observed differences in DC marker expression levels compared with healthy donor blood. Spleen and blood DC of deceased organ donors were very similar. In addition to the reduced frequency of CD16⁺ DC, they expressed lower CD16 levels, suggesting downregulation of CD16. Furthermore, CD141 expression was increased on most CD11c⁺ cDC, and CD11c expression was increased on CD141⁺ DC (Fig. 1). The total PBMC number per milliliter of blood as well as the proportion of DC were reduced in organ donor compared with healthy control blood (Fig. 1A, 1B, and data not shown).

We also analyzed spleen DC subsets from an organ donor after cardiac death, who was not treated with MP. The donor’s DC had a similar phenotype to brain-dead organ donors (Fig. 1D). However, the frequency of CD141⁺ cDC and the CD304⁺ pDC recovered from total splenocytes from cardiac death donors was markedly reduced, consistent with the recognized inferior quality of cardiac death tissues (30). Because of this, and because of the rarity of non–MP-treated donors, our focus was on DC from brain-dead organ donor spleens. Furthermore, the low frequency of DC in organ donor blood restricted further detailed comparisons of organ donor blood and spleen DC, necessitating further comparisons with DC from healthy donor blood.

In summary, when comparing the same donor, the phenotype of human blood and spleen DC was very similar, but altered by organ donor health status.

**Organ donor blood and spleen DC appear to have a partially activated phenotype**

We analyzed DC expression of surface activation markers (CD80, CD83, CD86, and CD40) in healthy donor blood and brain-dead organ donor spleen. Healthy donor blood DC subsets did not have detectable expression of these markers except for CD86, indicative of a resting phenotype (Fig. 2A). Brain-dead organ donor spleen CD1b/c⁺ and CD141⁺ cDC had higher expression of CD80 and HLA-DR compared with their blood counterparts (Fig. 2B). HLA-DR expression was also higher on spleen CD16⁺ DC. Thus, brain-dead organ donor spleen cDC appear to have an activated phenotype.

We hypothesized that the organ donor DC phenotype reflected DC activation resulting from release of proinflammatory mediators following brain death. To test whether proinflammatory

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**FIGURE 3.** Human blood and spleen DC have very similar patterns of transcription factor expression. Quantitative RT-PCR of transcription factors IRF4, IRF8, and ID2 relative to GAPDH in DC subsets isolated by FACS from healthy donor blood (CD1b/c⁺, CD141⁺, CD16⁺, CD304⁺; left panels) and brain-dead organ donor spleen (CD1b/c⁺, CD141⁺, CD16⁺, CD304⁺; right panels). Results are representative of two independent experiments.
mediators can induce a similar phenotype in healthy donor blood DC, FACS-sorted healthy donor blood DC subsets were stimulated overnight with a mixture of influenza virus protein, CD40L, IFN-α, and poly (I:C). This fully activated DC as expression of all activation markers tested was increased (Supplemental Fig. 3A). Interestingly, in vitro activation induced a similar DC marker expression pattern as observed in organ donor spleen DC (Supplemental Fig. 3B). These findings lend support to the idea that a proinflammatory milieu in the organ donor is responsible for the partially activated DC phenotype rather than the location of DC in the spleen.

**Human and mouse spleen DC subsets similarly express CD11b, SIRPα, and CLEC9A**

In mouse spleen, CD8<sup>-</sup> and CD8<sup>+</sup> cDC differentially express a number of cell surface markers. CD8<sup>-</sup> DC express CD11b and Sirpa (37) and CD8<sup>+</sup> cDC express Clec9A (20, 38). Mouse spleen pDC express intermediate levels of Sirpa. Analysis of these markers in human blood and spleen DC showed that CD11b/c<sup>+</sup> and CD16<sup>+</sup> cDC expressed high levels of SIRPα and CD11b, but not CLEC9A, similar to mouse CD8<sup>+</sup> cDC and mouse monocytes, respectively (20, 39, 40) (Fig. 2). In contrast, CD141<sup>+</sup> cDC expressed CLEC9A but low levels of SIRPα and CD11b (18–20), similar to mouse CD8α<sup>+</sup> cDC (Fig. 1) (20). CD304<sup>+</sup> pDC expressed intermediate levels of SIRPα and low levels of CD11b similar to mouse pDC.

Transcription factor IRF4, IRF8, and ID2 expression is identical in human blood and spleen, but IRF4 expression differs compared with mouse spleen DC subsets

We investigated other markers in human DC that in the mouse demarcate DC subsets. Mouse DC subsets differ in their development, which is controlled by transcription factors such as IFN regulatory factor (IRF)4, IRF8, and Id2. Mice lacking a functional IRF4 gene have almost no splenic CD4<sup>+</sup>CD8<sup>-</sup> cDC (41). In contrast, IRF8 is crucial in the development of mouse CD8<sup>+</sup> cDC and pDC (41). Id2 has been shown to be required for the development of mouse CD8<sup>+</sup> cDC (42) and human pDC (43) and is expressed in more mature human myeloid cells, including macrophages (44).

IRF4, IRF8, and Id2 expression in human DC subsets sorted from healthy donor blood and brain-dead organ donor spleen were analyzed by quantitative real-time PCR (Fig. 3). Overall, the pattern of transcription factor expression in human DC subsets correlated with that in mouse spleen DC. Human CD141<sup>+</sup> cDC and CD304<sup>+</sup> pDC expressed IRF8, similar to mouse CD8<sup>+</sup> and pDC. Human CD141<sup>+</sup> and CD16<sup>+</sup> cDC expressed Id2, similar to mouse CD8<sup>+</sup>, CD8<sup>+</sup> DC, and monocytes (G. Belz, personal communication). However, unlike mouse CD8<sup>+</sup> cDC, human CD11b/c<sup>+</sup> cDC lacked IRF4 expression.

**TLR expression is similar in human spleen and blood DC and differs from mouse spleen DC**

TLRs are expressed differentially by DC subsets and confer on DC the capacity to respond to specific pathogen-associated molecular patterns. TLR3 binds dsRNA and is expressed selectively by mouse CD8<sup>+</sup> cDC (45, 46). TLR4 binds bacterial LPS and is expressed at low levels by all mouse DC subsets and at high levels by monocytes. TLR7 binds ssRNA and is expressed by mouse CD8<sup>+</sup> cDC and pDC (45, 46). TLR9 binds bacterial CpG and is most highly expressed by mouse pDC, but is also expressed by other mouse cDC (37, 45). Expression of these TLRs in human DC was determined by quantitative real-time PCR. Human healthy donor blood and brain-dead organ donor spleen DC had very similar expression profiles (Fig. 4), with CD141<sup>+</sup> cDC expressing higher levels of TLR3 than other DC subsets, although splenic CD141<sup>+</sup> DC expressed relative lower TLR3 levels than did their blood counterpart. The pattern of TLR3 expression by human DC subsets correlated well with that of the mouse spleen DC counterparts. However, TLR4, TLR7, and TLR9 were less broadly expressed across human cDC subsets. In contrast to mouse DC, TLR9 was expressed exclusively by human pDC and not by any other human cDC subset in either blood or spleen. Neither TLR4 nor TLR7 were expressed by CD141<sup>+</sup> DC, and CD11b/c<sup>+</sup> DC expressed only low levels of TLR7 compared with pDC. These results suggest a stronger specialization of human DC subsets for the recognition of specific pathogen groups.

**Secretion of IL-12p70 by human DC subsets is not confined to CD141<sup>+</sup> cDC the proposed counterparts of mouse CD8<sup>+</sup> DC**

An important property of DC is their capacity to secrete cytokines in response to stimulation. Murine CD8<sup>+</sup> cDC secrete IL-12p70, which polarizes T cells to a Th1 response (6), whereas CD8<sup>+</sup> cDC secrete large amounts of inflammatory chemokines, including Mip1-α (5). pDC are the major secretors of type 1 IFN in response to viral stimulation (7–10). In mice, maximal production of IL-12p70 by cDC in vitro occurs after stimulation by a TLR9 agonist, together with the mixture of either IL-4, GM-CSF, and IFN-γ cytokines (47) or microbial stimuli together with anti-CD40 (48).
To avoid biasing the response of cDC subsets that differentially expressed TLR3, TLR4, and TLR7, we stimulated them for 24 h with CD40L and the cytokine mixture to ensure activation of all cDC rather than a particular subset (48–50). Because CD304+ pDC expressed very high levels of TLR9, they were stimulated with CpG-A and CpG-C.

IL-6 was highly secreted by both healthy donor blood and brain-dead organ donor spleen CD16+ DC (Fig. 5A), even in the absence of stimulation. After stimulation, IL-6 was also highly secreted by CD304+ blood pDC (Fig. 5A). Mip1-α and TNF-α were highly secreted by both CD16+ cDC and CD1b/c+ cDC from both spleen and blood (Fig. 5B, 5D). Again, these cytokines were highly secreted by blood CD304+ pDC. Although IL-12p70 is secreted mainly by CD8+ cDC in the mouse (47), in human blood and spleen all cDC secreted IL-12p70 when stimulated with CD40L and the cytokine mixture (Fig. 5C, 5F). CD304+ DC did not secrete IL-12p70. Blood but not spleen CD304+ DC were high secretors of IFN-α in response to TLR9 stimulation (Fig. 5E and data not shown). Human blood and spleen cDC had very similar cytokine secretion patterns, whereas spleen pDC secreted less cytokines overall than did blood pDC. Human DC cytokine secretion patterns were very similar to those of their mouse spleen DC counterparts, with the exception of IL-12p70, which in human DC under these conditions was secreted by all cDC subsets.

**Corresponding blood and spleen DC subsets have similar capacities to stimulate CD4+ T cell proliferation in an MLR**

Mouse DC subsets display differential capacities to stimulate T cells. CD8− and CD8+ cDC are potent stimulators of allogeneic CD4+ T cells compared with pDC (51). We tested human DC subsets from healthy donor blood and brain-dead organ donor spleen for their capacity to stimulate proliferation of CFSE-labeled allogeneic CD4+ T cells from human blood in an MLR. A similar hierarchy of T cell stimulatory capacity was observed with human blood and spleen DC subsets. CD304+ pDC were poor stimulators; among cDC, CD1b/c+ DC stimulated T cells most strongly followed by CD141+ DC; CD16+ DC were less efficient (Fig. 6).

Overall, blood DC from healthy donors were more efficient at eliciting T cell proliferation compared with their splenic counterparts from brain-dead organ donors. This was unexpected given the more activated status of the organ donor spleen DC. We hypothesized that the treatment of brain-dead organ donors with MP shortly before organ donation reduces the stimulatory capacity of their spleen DC. We compared spleen CD1b/c+ DC from two cardiac death organ donors, who had not been treated with MP, with blood CD1b/c+ DC from a healthy donor and spleen CD1b/c+ DC from an MP-treated brain-dead organ donor. CD1b/c+ DC from spleen donated after cardiac death and healthy donor blood had equal capacity to stimulate CD4+ T cell proliferation compared with the much weaker capacity of CD1b/c+ DC from MP-treated brain-dead organ donor spleen (Supplemental Fig. 4A). We also found that exposure of enriched healthy donor blood DC to MP in vitro strongly reduced their capacity for stimulation of CD4+ T cells by influenza Ag (Supplemental Fig. 4B).

**FIGURE 5.** Similar cytokine secretion patterns by human blood and spleen DC subsets. Healthy donor blood and brain-dead organ donor spleen CD1b/c+, CD141+, CD16+, and CD304+ DC were cultured at 1 × 10⁷/ml for 24 h in medium alone (unstimulated) or with CD40L (1 μg/ml), IFN-γ (40 ng/ml), GM-CSF (100 ng/ml), and IL-4 (40 ng/ml) for CD1b/c+ DC and CD16+ cDC, or with CpG A (1 μM) and CpG C (1 μM) for CD304+ pDC. The supernatant media were assayed by Immunoplex bead array. Results for (A) IL-6, (B) Mip1-α, (C) IL-12p70, (D) TNF-α, and (E) IFN-α are shown. F, IL-12p70 production by blood CD1b/c+ and CD141+ DC is shown as in C, with CD16+ DC and CD304+ DC omitted. Results are shown as mean (±SEM) of five independent experiments. BD, brain dead.
Cross-presentation capacity of human blood and spleen DC subsets

The ability to cross-present exogenous Ag on MHC class I proteins to CD8+ T cells distinguishes mouse spleen CD8+ from CD8- cDC. We therefore compared human healthy donor blood and brain-dead organ donor spleen DC for their capacity to cross-present influenza virus protein to a CD8+ T cell clone specific for an epitope in influenza virus matrix protein. CD8+ T cell activation was measured as IFN-γ secretion by an ELISPOT assay. Among blood DC, the CD1b/c+ subset activated the highest number of IFN-γ-producing CD8+ T cells (Fig. 7A). CD141+ DC activated less CD8+ T cells but were boosted by poly (I:C) (Fig. 7C). CD304+ pDC were also able to activate the CD8+ T cells but were not boosted by CD40L/IFN-α or poly (I:C) (data not shown). Spleen CD1b/c+ and CD141+ cDC as well as CD304+ pDC activated CD8+ T cells, although less efficiently than did blood DC (Fig. 7B). CD40L/IFN-α significantly boosted activation by spleen CD1b/c+ DC but not by any other subset (Fig. 7D). Poly (I:C) had no effect on any spleen DC subset, including CD141+ DC (Fig. 7D), consistent with the lower expression of TLR3 in spleen CD141+ DC than their blood counterpart. Different patterns of response were observed in healthy donor blood and brain-dead organ donor spleen DC (Fig. 7), with the former generally being more efficient. As in the MLR experiments above, exposure of DC to MP in vitro reduced their capacity to cross-present exogenous Ag and stimulate CD8+ T cells (Supplemental Fig. 4C).

Discussion

DC are important immunotherapeutic targets in cancer and autoimmune disease. Therapies that target DC have generally been developed using mouse models. Comparative knowledge of human blood versus lymphoid tissue DC, and in relationship to mouse DC, has been lacking but would greatly facilitate basic and applied studies of human DC. To our knowledge, our study is the first functional characterization of human spleen DC subsets. Additionally, we performed a comprehensive side-by-side comparison of the four different human spleen DC subsets as well as their counterparts in human blood.

The four DC subsets described in human blood were also present in human spleen and almost identical in phenotype, when compared in the same donor. Transcription factors and TLRs were identically expressed in the respective DC subsets in both blood and spleen, with the only exception being TLR3. Consistent with the phenotypic similarities, the functional capacities of human blood and spleen DC subsets for cytokine secretion, induction of allo- genetic CD4+ T cell responses, and cross-presentation to CD8+ T cells were similar. Overall, our findings reveal that differences...
between human blood and spleen DC are minimal. This is in
contrast to the mouse system where blood contains only the
immediate precursors of lymphoid tissue DC (28). Importantly,
however, note that the DC subset markers used were originally
identified as markers of blood DC subsets and may not necessarily
distinguish precursors from differentiated spleen resident DC.
This will be the subject of further investigation.

Differences were observed between healthy blood donor DC and
deceased organ donor blood and spleen DC. Activation marker
expression was increased and there were changes in DC subset
marker expression. Both MP treatment (52, 53) and hypoxia (52)
have been associated with a decrease in circulating CD16+CD14+ monocytes. In the present study, the two cardiac death donors
did not receive MP but still had reduced numbers of CD16+ DC, indicating that factors other than the MP treatment cause the
downregulation of CD16 in organ donors.

We showed that changes in DC marker expression as observed in
organ donor DC could be induced by activation in vitro, suggest-
ing that brain death-associated inflammation (36, 54, 55) may simi-
larly activate DC in vivo. Although activation markers were in-
creased on organ donor spleen DC, their expression levels were
lower than on fully activated DC. This could be due to treatment
of donors with MP, as we showed that treatment of DC with MP
in vitro dampened T cell stimulation capacity. Furthermore, spleen
DC from organ donors treated with MP were less potent in stimu-
ulating T cells than DC from non–MP-treated donors. We suggest
that treatment of organ donors with MP reduces the functional
maturation of DC despite brain death-induced inflammation.

We also showed that mouse spleen DC have counterparts in
human spleen with a very similar phenotype. Human spleen and
blood DC subsets expressed the surface markers CLEC9A, SIRPa,
and CD11b in the same pattern as their mouse spleen equivalents.
This is consistent with previous studies that proposed human blood
DC counterparts for mouse spleen DC subsets (17–21). The com-
parison of transcription factor and TLR expression revealed many
similarities but also some basic differences between mouse and
human DC subsets. IRF8 expression and TLR3 expression were
similarly distributed whereas differences were found for IRF4 and
TLR9. TLR9 is expressed by all mouse DC subsets (45, 46) but not
by any human cDC subset. Human DC TLR4 and TLR7 expression
patterns also differed from their mouse spleen counterparts. Taken
gether, these results suggest that there are differences in develop-
mental requirements and pathogen recognition capacities between
the proposed equivalent mouse and human DC subsets.

Our comprehensive side-by-side comparison of human DC sub-
sets showed a capacity of all human cDC to secrete IL-12p70 and
cross-present exogenous Ag to CD8+ T cells, which in mice are
hallmark functions of the CD8+ DC subset. IL-12p70 secretion
and cross-presentation by human DC subsets other than CD141+ DC
have been reported previously, but few studies compared other
human DC subsets to the CD141+ DC. A study by Skrzeczyńska-
Moncznik et al. (27) showed that human CD16+CD14+ mono-
cytes produce high amounts of IL-12p70, consistent with our
finding of IL-12p70 secretion by CD16+ blood DC. Jungbloed
et al. (24) stimulated CD1b/c+ DC with poly (I:C) and a cytokine
mixture but did not detect IL-12p70 secretion, whereas CD141+ DC
secreted IL-12p70 similarly as in our study. It is known that
IL-12p70 secretion by mouse and human DC varies depending on
optimal stimulation and cytokine environment (56). Poulin et al.
(25) found that IL-12p70 secretion was strongly enhanced by
coculture of CD141+ DC with Ag-stimulated T cells. Therefore,
varying results may be due to differences in the experimental
conditions used and each subset may require different stimuli.
Hochrein et al. (56) reported similar IL-12p70 secretion by total
human thymus-derived DC as we found in human blood and
spleen cDC, but >10-fold less than for mouse CD8α+ DC. Our
side-by-side comparison did not reveal greater IL-12p70 secretion
by any one human DC subset.

Similar to IL-12p70 secretion, the ability to cross-present ex-
ogenous Ag was not restricted to human CD141+ DC, as CD1b/c+
and CD16+DC, as well as pDC, were also able to cross-present.
Blood CD1b/c+ DC induced the strongest CD8+ T cell responses
in the absence of additional stimulation. Human pDC, monocyte-
derived DC, and pDC have previously been shown to cross-present
under certain conditions (57, 58), confirmed by more recent studies
(23, 24). In mice, monocyte-derived DC that differentiate in vivo
under inflammatory conditions have also been found to cross-
present (59) and to secrete IL-12p70 (60). Taken together, our
results indicate that mouse and human DC subsets are func-
tionally not fully equivalent. Spreading some functions across
more than one human DC subset may have had evolutionary ben-
efits, whereas differentially restricting expression of TLRs and
possibly other pattern recognition receptors may have been a
means of specializing responses to pathogens.

In conclusion, we identified and characterized four DC subsets
in human spleen from deceased organ donors. These share many
features with the DC subsets in human blood, including surface
marker expression, transcription factor and TLR expression, cy-
tokine secretion, and capacity to stimulate CD4+ and CD8+ T cells.
A modestly activated surface phenotype and reduced T cell stimu-
lation capacity of human spleen compared with blood DC sub-
sets could be accounted for by organ donor health status and not
by tissue location. The findings of this study therefore support the
validity of using blood DC from healthy donors to advance our
understanding of human DC biology. We also identified important
functional differences between phenotypically similar mouse and
human DC subsets that may be relevant to the development of DC-
based vaccines for human immunotherapy.

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