MAJOR ARTICLE

Normal Functional Capacity in Circulating Myeloid and Plasmacytoid Dendritic Cells in Patients with Chronic Hepatitis C

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Initial reports analyzing dendritic cell (DC) function in patients with hepatitis C virus (HCV) infection have been controversial. Here, we enumerate and characterize the function of circulating myeloid and plasmacytoid DCs. The results show lower percentages of myeloid DCs (0.62 vs. 0.83; \( P < 0.05 \)) and plasmacytoid DCs (0.11 vs. 0.34; \( P = 0.004 \)) in patients with chronic HCV infection than in healthy, non–HCV-infected individuals. Despite the lower numbers of circulating myeloid DCs present, no phenotypic or functional defects were identified. The lower percentage of plasmacytoid DCs resulted in decreased absolute interferon (IFN)–\( \alpha \) production; however, when analyzed on a per-cell basis, plasmacytoid DCs from HCV-infected patients generated levels of IFN–\( \alpha \) equivalent to those generated by DCs from healthy, non–HCV-infected individuals. Contrary to data from previous models (which attributed HCV pathogenesis to defects in the DC compartment), our data reveal functional DC subsets in patients with chronic HCV infection. These results are encouraging for DC-based HCV immunotherapy trials.

At present, ~200 million people worldwide are infected with hepatitis C virus (HCV). Current estimates indicate that 30% of infected individuals spontaneously resolve their infections [1]. Both resolution and protection correlate with the presence of HCV-specific T cell responses [2–4]. Of the 70% of HCV-infected individuals whose infection progresses to chronic HCV infection, pegylated interferon (IFN)–\( \alpha \) and ribavirin enable ~50% to sustain virological responses after treatment is discontinued [5, 6]. The roles that HCV-specific T cell responses and IFN–\( \alpha \) play highlight the importance of understanding dendritic cell (DC) function during HCV infection since myeloid DCs are required for priming T cells in vivo and since plasmacytoid DCs are the principle cell type responsible for endogenous IFN–\( \alpha \) production [7].

Although initial reports have shown impaired maturation and allostimulation of monocyte-derived DCs from patients with chronic HCV infection [8–10], our work in humans and recent studies in chimpanzees do not support these findings [11, 12]. Nonetheless, one caveat is that these studies focused on in vitro–derived DCs. To evaluate native circulating DCs, we took advantage of the ability to purify myeloid DCs, which make up 0.5%–1.0% of the peripheral blood mononuclear cells (PBMCs) and can be purified on the basis of expression of blood dendritic cell antigen (BDCA)–1 and lack of expression of M.1.A.

Potential conflicts of interest: none reported.

a C.M.R. and M.L.A. contributed equally to this work.

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Table 1. Characteristics of patients with chronic hepatitis C virus (HCV) infection in the present study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, years</th>
<th>Sex</th>
<th>Genotype</th>
<th>ALT/AST score</th>
<th>HCV load, IU/mL (×10^4)</th>
<th>Histological finding</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1181</td>
<td>55</td>
<td>M</td>
<td>NA</td>
<td>60/89</td>
<td>62.5</td>
<td>NA</td>
<td>Naive</td>
</tr>
<tr>
<td>347</td>
<td>51</td>
<td>F</td>
<td>1a</td>
<td>207/145</td>
<td>431</td>
<td>Grade 3, stage 2</td>
<td>Peg-IFN/RBV</td>
</tr>
<tr>
<td>1142</td>
<td>46</td>
<td>F</td>
<td>1b</td>
<td>40/43</td>
<td>128</td>
<td>Grade 2, stage 3–4</td>
<td>Naive</td>
</tr>
<tr>
<td>1060</td>
<td>38</td>
<td>F</td>
<td>1</td>
<td>129/77</td>
<td>42.2</td>
<td>Stages 1–2</td>
<td>Naive</td>
</tr>
<tr>
<td>107</td>
<td>33</td>
<td>M</td>
<td>1a</td>
<td>74/28</td>
<td>2.5</td>
<td>Grade 3, stage 3</td>
<td>Peg-IFN/RBV (treated in 2003)</td>
</tr>
<tr>
<td>1128</td>
<td>57</td>
<td>F</td>
<td>1b</td>
<td>51/NA</td>
<td>45</td>
<td>Stage 2</td>
<td>Naive</td>
</tr>
<tr>
<td>1127</td>
<td>54</td>
<td>F</td>
<td>2a/c</td>
<td>138/93</td>
<td>14.6</td>
<td>Grade 1, stage 1</td>
<td>Naive</td>
</tr>
<tr>
<td>959</td>
<td>29</td>
<td>M</td>
<td>2b</td>
<td>235/87</td>
<td>385</td>
<td>Grade 3, stage 3</td>
<td>Naive</td>
</tr>
<tr>
<td>967</td>
<td>64</td>
<td>M</td>
<td>1a</td>
<td>48/31</td>
<td>NA</td>
<td>Grade 3, stage 3</td>
<td>Naive</td>
</tr>
<tr>
<td>1133</td>
<td>47</td>
<td>M</td>
<td>1a</td>
<td>48/42</td>
<td>182</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>840</td>
<td>43</td>
<td>M</td>
<td>1b</td>
<td>66/40</td>
<td>222.2</td>
<td>Grade 1, stage 3</td>
<td>Naive</td>
</tr>
<tr>
<td>501</td>
<td>58</td>
<td>F</td>
<td>2b</td>
<td>29/30</td>
<td>&gt;100</td>
<td>Grade 3, stage 1</td>
<td>Naive</td>
</tr>
<tr>
<td>522</td>
<td>60</td>
<td>F</td>
<td>1b</td>
<td>82/60</td>
<td>27.7</td>
<td>Grade 2, stage 2</td>
<td>Naive</td>
</tr>
<tr>
<td>573</td>
<td>52</td>
<td>M</td>
<td>1a</td>
<td>24/33</td>
<td>NA</td>
<td>Grade 2, stage 2</td>
<td>Peg-IFN/RBV (treated in 2003)</td>
</tr>
<tr>
<td>1166</td>
<td>46</td>
<td>M</td>
<td>1α/b</td>
<td>63/51</td>
<td>872</td>
<td>NA</td>
<td>Naive</td>
</tr>
<tr>
<td>1161</td>
<td>70</td>
<td>F</td>
<td>1α</td>
<td>204/113</td>
<td>264</td>
<td>Grade 2, stage 1</td>
<td>Naive</td>
</tr>
<tr>
<td>400</td>
<td>53</td>
<td>M</td>
<td>1α</td>
<td>104/106</td>
<td>13</td>
<td>Grade 3, stage 4</td>
<td>Naive</td>
</tr>
</tbody>
</table>

NOTE. A patient with chronic HCV infection was defined as an individual with a detectable HCV load and sustained liver injury for >6 months, as monitored by liver function tests (alanine aminotransferase [ALT]/aspartate aminotransferase [AST]) and/or liver biopsy. IFN, interferon; NA, not available; PEG, pegylated; RBV, ribavirin.

* HCV load defined by quantitative polymerase chain reaction at the time of collection of blood samples (Roche Amplicor). Data were not available for patients 967 and 573; prior data confirmed that they had chronic HCV infection.

* Histological criteria defined as in [16].

* Indicates leukapheresis sample.

* Indicates fresh sample used for analysis of IFN-α production; no marking indicates a frozen sample.

populations of interest and determination of functional activity (e.g., production of IFN-α) [15]. Here, we provide a careful analysis of both cell populations in patients with chronic HCV infection and conclude that there are no phenotypic or functional defects in the myeloid DC subset and no decrease in the amount of IFN-α produced on a per-plasmacytoid DC basis.

PATIENTS, MATERIALS, AND METHODS

Patients with chronic HCV infection. Study samples were collected from patients in accordance with protocols approved by the Weill Medical College of Cornell University and The Rockefeller University Hospital Institutional Review Board, and all patients gave written, informed consent. HCV infection was confirmed on the basis of serological test results and/or plasma HCV titers. Chronic HCV infection was defined as HCV infection and sustained liver injury for >6 months, as monitored by liver function tests and/or liver biopsy. Pegylated IFN/ribavirin therapy had been discontinued at least 6 months before collection of samples. In addition, patients had not received any immune modulators or suppressors within 30 days of study entry. All patients tested negative for HIV, syphilis, and hepatitis B virus within 30 days of study entry and were free of uncontrolled medical illness (including decompensated cirrhosis and any rheumatological or immunological disease). Samples were collected from 17 patients with chronic HCV infection (8 women and 9 men; median age, 52 years; age range, 29–70 years) (table 1). Samples were also collected from 17 healthy, non–HCV-infected individuals. Sustained virological responders (SVRs) (n = 6) were HCV RNA negative 6 months after completion of pegylated IFN/ribavirin therapy. PBMCs were also collected from patients with non-HCV liver disease (n = 7), including hepatitis B, granulomatous hepatitis, and cirrhosis.

Isolation and preparation of cells. PBMCs were isolated from whole blood by sedimentation over Ficoll-Hypaque (Amersham Pharmacia). Myeloid DCs were isolated by use of a BDCA-1 Isolation Kit (Miltenyi Biotec). Mature myeloid DC cultures were generated after purification with 50 ng/mL tumor necrosis factor (TNF)–α (AlexisBiochemicals) and 0.1 mmol/L prostaglandin E2 (Sigma) for 36–48 h [17].

Cell enumeration. Flow-cytometric analysis was used to enumerate different hematopoietic lineages in PBMCs by use of a Blood Dendritic Cell Enumeration Kit (Miltenyi Biotec). Briefly, scatter properties of the cells, as well as staining with CD19 and CD14 monoclonal antibodies (MAbs), enabled enumeration of B cells and monocytes, respectively. Simultaneous labeling with anti-CD1c (BDCA-1) and anti–BDCA-2 MAbs allowed identification of CD19+ cells and enumeration of the BDCA-1+ myeloid DC and the BDCA-2+ plasmacytoid DC populations. Absolute numbers of the respective cell populations.
Figure 1. Enumeration of dendritic cells (DCs) in chronic hepatitis C virus (HCV) infection. A, Flow-cytometric-based enumeration strategy. Peripheral blood mononuclear cells were gated in R1 on the basis of forward scatter (FSC)/side scatter (SSC). B cells and monocytes were identified by CD19 and CD14 Cy5 labeling, respectively, and were differentiated on the basis of SSC profile. B cells were gated in R2, and monocytes were gated in R3. CD19<sup>−</sup> and CD14<sup>low</sup> cells were gated in R4 and used to enumerate blood dendritic cell antigen (BDCA)-1 phycoerythrin (PE) myeloid DCs (R5) and BDCA-2 fluorescein isothiocyanate (FITC) (R6). B, Percentages of monocytes and B cells, graphed for healthy, non–HCV-infected individuals (triangles) and patients with chronic HCV infection (circles). The percentages of myeloid DCs (BDCA-1<sup>+</sup>) and plasmacytoid DCs (BDCA-2<sup>+</sup>) in healthy, non–HCV-infected individuals, patients with chronic HCV infection (HCV), sustained virological responders (SVRs) (squares), and patients with non-HCV liver disease (diamonds) are also displayed graphically. The Mann-Whitney U test was used to generate P values for comparison of healthy, non–HCV-infected individuals (n = 17) and patients with chronic HCV infection (n = 17).

were normalized to the total number of PBMCs collected, to obtain the relative percentage of each cell type.

**Allogeneic mixed leukocyte reaction.** A total of 2 × 10<sup>5</sup> purified T cells/well were plated with myeloid DCs, at indicated dilutions, in 5% pooled human serum (Labquip LTD). Cultures were incubated for 4–5 days at 37°C and pulsed with 1 μCi [<sup>3</sup>H]-thymidine during the last 14–16 h of culture. [<sup>3</sup>H]-thymidine incorporation was measured by use of a liquid scintillation counter (Packard Topcount). The allogostimulatory index was generated by dividing the observed proliferation by...
Figure 2. Normal phenotype and function of dendritic cells (DCs) in patients with chronic hepatitis C virus (HCV) infection. A, Fluorescence-activated cell sorter phenotype of myeloid DCs (blood dendritic cell antigen [BDCA]–1 +), shown for a representative patient with chronic HCV infection (1181). Analysis was performed after CD19 depletion and BDCA-1–positive selection (90% purity; data not shown). Thick black line, Myeloid DCs plus tumor necrosis factor (TNF)–α; gray line, myeloid DCs; thin black line, isotype control. B, Allostimulatory responses for myeloid DCs purified from 4 patients with chronic HCV infection and 3 healthy, non–HCV-infected individuals. BDCA-1–purified cells alone or cells exposed to TNF-α were placed into coculture with allogeneic T cells and cultured for 4–5 days. Proliferation was monitored by [3H]-thymidine incorporation, and data were normalized to the average maximum stimulation by mature myeloid DCs, as described in Patients, Materials, and Methods. White circles, Myeloid DCs; black circles, myeloid DCs plus TNF (patients with HCV); white triangles, myeloid DCs; black triangles, myeloid DCs plus TNF (healthy individuals). Average proliferation responses across the patient populations are indicated by the lines. C, Interferon (IFN)–α production in the supernatant of peripheral blood mononuclear cells (PBMCs) stimulated with 50 HAU/mL influenza virus or 5 μg/mL CpG-2216, evaluated by ELISA. Median values are indicated by the lines, and P values were determined by use of the Mann–Whitney U test. D, Data from the ELISA, normalized on the basis of the respective percentage of plasmacytoid DCs present in the PBMC fraction showing production on a per–plasmacytoid DC basis. P values were determined by use of a Welch-corrected unpaired t test. E, Plasmacytoid DCs identified in the PBMC population by use of the lineage-specific markers BDCA-4 allophycocyanin (APC) and BDCA-2 fluorescein isothiocyanate (FITC) (gate R1). Intracellular IFN-α phycoerythrin (PE) staining in unstimulated or stimulated (50 HAU/mL influenza virus) conditions was measured by flow-cytometric analysis, and the percentage of IFN-α + plasmacytoid DCs is represented in a dot plot. Patient 1166 represents a donor with chronic HCV infection who had normal plasmacytoid (BDCA-2 +) numbers (0.62%). Patient 1138 represents a donor with chronic HCV infection who had low plasmacytoid (BDCA-2 +) numbers (0.05%). NL 3 was a non–HCV-infected individual. F, The percentage of plasmacytoid DCs responding to stimulation with influenza virus and producing IFN-α in 6 patients with chronic HCV infection and 4 healthy, non–HCV-infected individuals. The lines indicate the average percentages of IFN-α + plasmacytoid DCs across the respective patient population tested. FSC, forward scatter.

Determination of IFN-α production by use of intracellular cytokine staining and an ELISA. PBMCs were resuspended in Dulbecco’s MEM with 10% fetal bovine serum, at 5 × 10⁷ cells/mL, and stimulated with either media alone, 50 hemagglutination units (HAU)/mL A/PR8/1976, or 5 μg/mL CpG (2216, 5′-GGGGACGATCGTGGGGGG-3′). Cultures were incubated for 5 h, harvested, fixed by use of a Cytoperp/Cytofix kit (BD Pharmingen), and stained with anti–IFN-α (MMHA-11; PBL Laboratories) and then with goat anti-mouse phycoerythrin (PE) (Jackson Immunochemical). BDCA-2 fluorescein isothiocyanate and BDCA-4 allophycocyanin antibodies were
used to label plasmacytoid DCs in the PBMC fraction, after stimulation. To measure the quantity of total IFN-α produced, supernatants from parallel cultures were collected after 24 or 48 h and were assayed by use of a human IFN-α ELISA (PBL Laboratories). Importantly, all PBMC samples used for IFN-α assays were freshly prepared and used within 12 h, since IFN-α production was significantly affected by freeze-thaw (>10-fold decrease in IFN-α production in parallel samples). IFN-α production was normalized per plasmacytoid DC on the basis of the percentage of plasmacytoid DCs in the PBMC fraction.

**Statistics.** Two-tailed nonparametric comparisons (Mann-Whitney U test) were used to calculate $P$ values for differences in numbers of myeloid DCs and plasmacytoid DCs, as well as total IFN-α production by PBMCs, since this method allows for generation of data with a non-Gaussian distribution. $P$ values for normalized data were calculated by use of a 2-tailed parametric comparison (Welch-corrected unpaired $t$ test).

**RESULTS AND DISCUSSION**

To enumerate the different hematopoetic lineages in PBMCs, we used flow-cytometric analysis. By use of the scatter properties of the cells (size and granularity), as well as staining with anti-CD19 and anti-CD14 MAb, it was possible to enumerate B cells and monocytes (figure 1A). Cells were simultaneously labeled with anti-CD1c and anti–BDCA-2 MAb, thus allowing selection of CD19 cells and enumeration of the BDCA-1+ myeloid DC and the BDCA-2+ plasmacytoid DC populations (figure 1A). Absolute numbers of the respective cell populations were normalized to the total number of PBMCs collected, to obtain the relative percentage of each cell type (figure 1B). De-
spite the greater heterogeneity in the percentage of B cells in patients with HCV infection seen here as well as in previous studies [18], we observed no statistical difference in the median percentage of B cells or monocytes. We did, however, reveal a small but marginally significant difference in the percentage of myeloid DCs between patients with chronic HCV infection and healthy, non–HCV-infected individuals (0.62 vs. 0.83; \( P = .05 \)). In addition, the data indicate a reduction in the percentage of plasmacytoid DCs in patients with chronic HCV infection (0.11 vs. 0.34; \( P = .004 \)), compared with those from healthy, non–HCV-infected individuals. Although potentially important to disease progression, this observation is not specific for chronic HCV infection. We found lower numbers of DCs in SVRs \( (n = 6 \) ) and patients with non–HCV liver disease \( (n = 7 \) ). Low numbers of plasmacytoid DCs have also been found in patients with HIV/AIDS [19].

To isolate myeloid DCs and evaluate their functional characteristics, large numbers of PBMCs were collected by leukapheresis from 4 patients with chronic HCV infection. Surface expression of phenotypic markers was measured by flow-cytometric analysis and indicated a phenotype suggestive of immature DCs—CD14\(^+\), CD25\(^+\), CD40\(^+\), CD83\(^{low}\), CD86\(^{intermediate}\), and HLA-DR\(^{intermediate}\) (figure 2A). After exposure of myeloid DCs to TNF-\( \alpha \), up-regulation of the maturation markers CD25, CD40, CD83, CD86, and HLA-DR was observed (figure 2A). This up-regulation correlated with a functional change in allostimulatory potential, which is also characteristic of mature DCs (figure 2B). Of note, the phenotype and priming capacity of myeloid DCs from patients with chronic HCV infection were equivalent to the capacities of those from healthy, non–HCV-infected individuals (figure 2B).

We next addressed the function of plasmacytoid DCs from patients with chronic HCV infection. In humans, plasmacytoid DCs produce robust amounts of IFN-\( \alpha \) after engagement of Toll-like receptor (TLR) 7 (a receptor for ssRNA) or TLR9 (a receptor for CpG motifs) [20]. We stimulated the PBMCs with live influenza virus or CpG-2216 and measured IFN-\( \alpha \) production by ELISA (figure 2C). Notably, IFN-\( \alpha \) production was lower in patients with chronic HCV infection than in healthy, non–HCV-infected individuals when PBMCs were stimulated with 50 HAU/mL influenza virus (3.9 vs. 12.3 ng/mL; \( P = .07 \)) or CpG-2216 (4.9 vs. 26.1 ng/mL; \( P = .04 \)). However, when normalized to the respective percentage of plasmacytoid DCs in the PBMC sample, the difference in IFN-\( \alpha \) production was no longer statistically significant between patients with chronic HCV infection and healthy, non–HCV-infected individuals for either influenza virus (1.3 vs. 1.9 pg/mL) or CpG (2.0 vs. 2.9 pg/mL) (figure 2D). These findings suggest that, although lower in number, the plasmacytoid DCs are unimpaired in their production of IFN-\( \alpha \) in response to TLR ligands.

To verify IFN-\( \alpha \) production by plasmacytoid DCs from HCV-infected patients and to evaluate the percentage of IFN-\( \alpha \)-producing plasmacytoid DCs, we assayed stimulated PBMCs for intracellular IFN-\( \alpha \) by flow-cytometric analysis, after 5 h of stimulation with influenza virus or CpG-2216 (figure 2E). Only the plasmacytoid DCs produced measurable IFN-\( \alpha \) under these conditions, and, by gating this population, we could assess the percentage of IFN-\( \alpha \)-producing plasmacytoid DCs (figure 2E). The data indicate that, despite the decreased percentage of plasmacytoid DCs in the circulating blood, plasmacytoid DCs respond equally well to influenza virus and CpG motifs on a per-cell basis (figure 2D–2F).

The role that DCs play in chronic HCV infection has been controversial. Although several studies initially reported dysfunction in monocyte-derived DCs [8–10], our prior work argues for fully functional DCs in patients with chronic HCV infection [11]. This controversy extends to circulating myeloid DCs; however, no direct mechanism for the putative DC dysfunction has been demonstrated [21, 22]. Furthermore, these reports do not account for the HCV specificity of the immune defect. Similar to those of several recent studies, our data support the finding of lower percentages of myeloid DCs and plasmacytoid DCs in PBMCs from patients with chronic HCV infection [22, 23]. However, on the basis of the isolation and characterization of myeloid DCs (figure 2A and 2B), as well as the single-cell analysis of plasmacytoid DCs (figure 2D–F), we conclude that there is no global dysfunction in these cell populations in vivo as a result of chronic HCV infection. Moreover, these findings support the use of autologous DCs as an effective strategy for HCV immunotherapy [12] as well as provide a rationale for current strategies aimed at stimulating endogenous IFN-\( \alpha \)-production through the use of plasmacytoid DC–stimulating agents, such as CpG [24].

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References


