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Dendritic Cell Inhibition Is Connected to Exhaustion of CD8+ T Cell Polyfunctionality during Chronic Hepatitis C Virus Infection

Ian Gaël Rodrigue-Gervais,*,†,‡ Hawley Rigby,* Loubna Jouan,*§ Dominike Sauvé,*,† Rafick-Pierre Sékaly,*,†,‡ Bernard Willems,*§ and Daniel Lamarre,*,†,§,¶

Although chronic viral infections have evolved mechanisms to interfere with aspects of pathogen recognition by dendritic cells (DCs), the role that these APCs play in virus-specific T cell exhaustion is unclear. Herein we report that NS3-dependent suppression of Toll/IL-1 domain-containing adapter-inducing IFN-β– and IFN-β promoter stimulator-1– but not MyD88-coupled pathogen-recognition receptor–induced synthesis of proinflammatory cytokines (IL-12 and TNF-α) from DCs by hepatitis C virus (HCV) is a distinctive feature of a subgroup of chronically infected patients. The result is decreased CD8+ T cell polyfunctional capacities (production of IFN-γ, IL-2, TNF-α, and CD107a mobilization) that is confined to HCV specificities and that relates to the extent to which HCV inhibits DC responses in infected subjects, despite comparable plasma viral load, helper T cell environments, and inhibitory programmed death 1 receptor/ligand signals. Thus, subjects in whom pathogen-recognition receptor signaling in DCs was intact exhibited enhanced polyfunctionality (i.e., IL-2 secretion and CD107a). In addition, differences between HCV-infected patients in the ability of CD8+ T cells to activate multiple functions in response to HCV did not apply to CD8+ T cells specific for other immune-controlled viruses (CMV, EBV, and influenza). Our findings identify reversible virus evasion of DC-mediated innate immunity as an additional important factor that impacts the severity of polyfunctional CD8+ T cell exhaustion during a chronic viral infection. The Journal of Immunology, 2010, 184: 3134–3144.

Chronic hepatitis C (CHC) is a liver-borne infectious disease that remains a global health threat. Its persistence is due to the failure of the host’s immune system to effectively clear the infection and generate protective cellular immunity. Marked quantitative and qualitative defects in this response have been described in patients (1): hepatitis C virus (HCV)-specific CD8+ T cells are nonresponsive to viral Ag and persist in a non-functional exhausted state, unable to produce important antiviral and immune-stimulatory cytokines, such as IL-2 and TNF-α; lyse virally infected cells; or proliferate. As documented for other persistent infections, inadequate CD4 help is widely perceived as a principal factor responsible for the exhaustion of CD8s and their failure to afford protection against the highly mutable HCV genome (2). Yet despite extensive efforts, it is not clear how virus-specific cellular immunity fails in most individuals. Any model of immune evasion brought forward is required to explain defects in cellular immunity that are exquisitely HCV-specific in persistently infected individuals who have otherwise normal immune functions to unrelated pathogens (3). One model that has recently attracted much attention stems from the discovery that the inhibitory programmed death-1 (PD-1), a cell-surface molecule that marks exhausted T cells, is generally upregulated on pathogen-specific CD8+ T cells in chronic viral diseases, including HCV (4, 5). Because the PD-1/programmed death ligand 1 (PD-L1) axis operates primarily under conditions of sustained high levels of Ag stimulation and inadequate help (6) to turn off Ag-specific CD8 to prevent immune-mediated damage, chronic pathogens end up taking advantage of this inhibitory pathway to establish persistence in the host. This negative regulator has become a therapeutic target of choice because Ab blocking of PD-L1 reinvigorates exhausted CD8 cells to control chronic SIV infection in macaques (7). However, PD-1 expression patterns alone do not conclusively predict the clinical outcomes of HCV infection (8). Functional CD8 effectors upregulate PD-1 during the first weeks of an acute viral infection, and some of these cells end up forming functional memory once the infection is cleared, despite continued expression of inhibitory receptors, albeit at lower levels (9). Thus, it is unclear whether inhibitory receptors cause T cells to become exhausted in the first place, indicating that different and additional signals, distinct from those relayed by PD-1–like molecules, modulate CD8+ T cell exhaustion as well.

One such signal could come from dendritic cells (DCs), because they play a pivotal role in activating and tuning the adaptive immune system.
arm. DCs survey, decapit and, quickly respond to pathogen-expressed danger stimuli through their pattern-recognition receptors (PRRs), whereby they become licensed to present components of pathogens to circulating T cells (10). However, presentation of viral Ag by DCs may prove to be a double-edged sword: to induce HCV-specific immune responses it is necessary for DCs to interact with HCV to deliver Ags and appropriate signals to T cells, leaving the HCV-presenting DCs susceptible to functional alterations by the virus. Proteolytic cleavage of adaptors Toll/IL-1 domain-containing adapter-inducing IFN-β (TRIF) and IFN-β promoter stimulator (IPS)-1 by HCV’s NS3/4A serine protease suppresses the activation of innate immunity by well-characterized PRRs, TRL3 and retinoic acid-inducible gene (RIG)-I, respectively (11–13). Pertinently, HCV RNA density-dependent attenuation of IL-12 and TNF-α production by myeloid DCs (MDCs) after TRL3 and TRLR4 stimulation, both TRIF-dependent pathways, was reported to occur in HCV-infected patients versus airmemics (14). A number of investigators independently reported similar impairment of PRR-induced synthesis of IL-12, TNF-α, and IFN-β from MDCs in every CHC cohort examined (15, 16). However, exactly how this impairment of PRR sensing in DCs factors into the control of HCV infection remains unclear for several reasons. In particular, no virus-induced mechanism leading to evasion of PRR recognition in DCs has been identified in patients. Even less is known about how DC sensing of pathogens during chronic infections can translate into polyvalent CD8 immunity in humans. Many studies in mice have defined the mechanisms of PRR-mediated control of adaptive immunity orchestrated by DCs to induce effective host resistance to pathogens (see review in Ref. 17). What has not been clear is whether the amount of activation of DCs results in better T cell effector functions in settings of chronic infection in which repeated Ag stimulation, lack of CD4 help, and PD-1/PD-L1 and other inhibitory signals are believed to drive T cells to exhaustion. It is also suggested that DC-tropic viruses that have evolved means to impair the function of these cells to enhance their chance to persist and escape immunosurveillance must be immunosuppressive in patients; erosion of T cell specificities unrelated to HCV is rarely a characteristic of CHC (3). Finally, in vitro experiments have not unambiguously demonstrated that PRRs on DCs are impaired by HCV infection (18). Thus, it is difficult, in that context, to establish whether impaired DC functions can have a detrimental impact on the functions of pathogen-specific T cells, independently of those effects ascribed to known factors (e.g., PD-1 expression and lack of CD4 help) and, additionally, without affecting unrelated T cell specificities (19).

Using complementary approaches, we addressed whether HCV-induced defects in PRR-mediated activation of DCs impacts the severity of HCV-specific CD8+ T cell exhaustion, without otherwise compromising the immune competence of the host. To this end, we established the molecular basis underlying the impairment of PRRs on MDCs in CHC and dissociate the impact that this defect has on polyfunctional CD8 immune exhaustion under conditions of similar plasma viral loads (PVLs), helper T cell environment, and PD-1/PD-L1 inhibitory signals. Ultimately, our findings reveal that multifunctional CD8+ effector T cells that possess the ability to produce IL-2 and degranulate can persist when DCs prove not to be inhibited by HCV. Moreover, HCV-induced defects in DCs are not associated with erosion of unrelated T cell specificities.

Materials and Methods

Study subjects

Patients with chronic HCV infection (viremics), spontaneous resolution of HCV, or sustained virological response to HCV therapy (SVR) were enrolled into a longitudinal study by the hepatology experimental unit of Hôpital Saint-Luc (Table I). All samples used in this study were from patients not receiving pegylated IFN-α2a and ribavirin therapy. Clinical protocols conformed to ethical guidelines of the authors’ institutions, and all subjects gave written informed consent. Patients 28–31 and 35 were described in a separate report (14). Viremics were grouped into cytokine profile-defective (CP-D) versus cytokine profile-normal (CP-N) DC function for LPS and polynosinic:polycytidylic acid [poly(L)C] stimulatory conditions at study entry (see FACS analysis and Table I). Control healthy subjects were documented to be hepatitis B virus-, HCV-, and HIV-1–seronegative. PVL was measured at the Laboratoire de Santé Publique du Québec. Blood was collected in heparin preparation tubes (Vacutainer Systems, BD Biosciences, Franklin Lakes, NJ). PBMCs were obtained by standard Ficoll-Paque density gradient centrifugation and cryopreserved at −140°C at 10^9 cells/ml in FCS (Sigma-Aldrich, St. Louis, MO) containing 10% DMSO until use.

PRR agonists and peptides

The following PRR agonists were used at the indicated final titrated concentrations measured at the Laboratory of Santé Publique du Québec. Blood was collected in heparin preparation tubes (Vacutainer Systems, BD Biosciences, Franklin Lakes, NJ). PBMCs were obtained by standard Ficoll-Paque density gradient centrifugation and cryopreserved at −140°C at 10^9 cells/ml in FCS (Sigma-Aldrich, St. Louis, MO) containing 10% DMSO until use.

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HCV RT-quantitative PCR

Cell-associated HCV RNA was quantified, as previously described (14). Samples with cell-associated viral load (CVL) values <2.0 log_10 copies × 10^6 PBMC were treated as negative and assigned a value of 100 RNA molecules for statistical analyses.

Activation and staining of cells

All cryopreserved PBMC samples were thawed in the presence of 50 U/ml Benzonase (Novagen, Madison, WI). For MDC assay, samples were transferred to 96-well plates (1.25 × 10^5 cells/well) and rested for 12 h in complete RPMI medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10 mM HEPES; referred to as R10) at 37°C, in 5% CO2. Staining PRR agonists (pretreated), brefeldin A (10 μg/ml final concentration; Sigma-Aldrich), and, in some cases, NS3 protease (1 μM; BILN2061), NS5B polymerase (10 μM; VIR235), viral inhibitors, or vehicle alone (0.1% DMSO) were added for 5.5 h at 37°C. A negative control containing PBMCs from the same individuals, but with no added peptide, and a staphylococcal enterotoxin B positive control, to ensure that cells were responsive (0.2 μg/ml final concentration; Toxin Technology, Sarasota, FL), were included for all samples in each assay. Following incubation, cells were washed in bright yellow dye ViViD for 30 min in the dark at 4°C. After intracellular staining for CD3, IFN-γ, IL-2, TNF-α, and CD154 (in the case of CD4), cells were fixed in PBS containing 1% formaldehyde and samples in each assay. Following incubation, cells were washed in bright yellow dye ViViD for 30 min in the dark at 4°C. After intracellular staining for CD3, IFN-γ, IL-2, TNF-α, and CD154 (in the case of CD4), cells were fixed in PBS containing 1% formaldehyde and stored at 4°C in the dark until FACS analysis (performed within 24 h). CFSE assays with PD-L1 blockade were performed as described previously (21), using a final concentration of 1 μg/ml peptide and 10 μg/ml anti-PD-L1 (clone MIH5, eBioscience, San Diego, CA). Cells were incubated for 7 d, restimulated with peptides (2 μg/ml), and processed for FACS as above.
**FACS analysis**

Data were acquired on an LSRII equipped for the detection of 11 fluorescent parameters. A minimum of 1.0 × 10^6 and 1.25 × 10^6 total PBMCs was collected by FACS for each DC and Ag-specific T cell sample, respectively. Data analysis was performed using FACS DiVa version 5.0.2 software. Initial gating of each sample set used a forward scatter (FSC) area versus an FSC-height plot to gate out cell aggregates. Analysis of MDCs involved CD3^+^, CD19^+^, and CD8^+^ lymphocyte exclusion using an AmCyan dump channel; the remaining events were subjected to an HLA-DR versus a side scatter-height gate to isolate MHC-II^+^ cells. Following this, MDCs were selected by sequentially gating on CD45^+, CD11c^+, CD14^−^, and CD16^−^ cells to generate mean fluorescence intensity (MFI) values for each cytokine in this cell type. Cytokine expression profile (CP) hierarchical clustering analyses of viremic patients into CP-D and CP-N were done as described (14), using the geometric MFI of MDCs positive for IL-12 and TNF-α for LPS- and poly(1C)-stimulatory conditions at study entry (referred to as week 0).

CD8^+^ T cells were selected by gating out CD14^+, CD19^+^, and dead cells (Pacific Blue dump channel) and gating on small (FSC and side scatter properties) CD3^+, CD4^+^, and CD8^+^ lymphocytes. All observed frequencies were normalized to the CD8 memory subset. To establish a precise definition of positive responses, net (i.e., minus background) CD8^+^ T cell frequencies were adopted as cutoff points for a positive response to a peptide pool, and patients were considered to have positive HCV responses if they were above the two-SD thresholds for any one of the aforementioned functions. Because HCV proteins were analyzed in parts, a positive response by these above the two-SD thresholds for any one of the aforementioned functions. Because HCV proteins were analyzed in parts, a positive response by these properties) CD3^+, CD4^+^, and CD8^+^ lymphocytes. All observed frequencies were normalized to the CD8 memory subset. To establish a precise definition of positive responses, net (i.e., minus background) CD8^+^ T cell responses to peptide pools in HCV-seronegative healthy subjects (n = 10) were analyzed; these formed a normal distribution, with a mean of 0 (cytokines) and 0.01 (CD107a) and two SDs equaling 0.01% for CD8^+^ IFN-γ, IL-2, and TNF-α analyses or 0.03% for CD107a. These two SD frequencies were adopted as cutoff points for a positive response to a peptide pool, and patients were considered to have positive HCV responses if they were above the two-SD thresholds for any one of the aforementioned functions. Because HCV proteins were analyzed in parts, a positive response by these criteria by any peptide submix constituted an HCV-specific response, and if one or more such positive responses were identified, these responses were summed to calculate a total Ag-specific response frequency. Boolean combinations of single-function gates (Fig. 3A) were created to determine the frequency of each response based on all possible combinations (up to 15) of cytokines and CD107a expression. A lower threshold, corresponding to two SDs above healthy donor background, was built for each specific functional combination, and values below this threshold were set to zero. Total frequencies of virus-specific CD8^+^ T cells were calculated by summing the frequencies within each unique combination of functions (counting each responding cell only once). A similar procedure was used to analyze CD4 responses to Core, NS3, NS4A/B, and NS5B.

**Statistics**

Statistical analysis was performed using the Vassar Web site (http://faculty.vassar.edu/lowry/VassarStats.html) and the GraphPad Prism software v3.0 statistical package. All tests were two-tailed, and p values < 0.05 were considered significant.

**Results**

A subgroup of viremic patients (cluster CP-D) has MDCs that exhibit a long-lasting functional defect restricted to their TRIF- and IFN-β promoter stimulator-1–dependent PRR-sensing activities

We reported previously that impaired PRR microbial sensing by DCs was a distinctive feature of a subgroup of patients chronically infected with HCV (CP-D) (14). To characterize the HCV-induced mechanism underlying this attenuation of PRR-mediated innate sensing, 20 viremic patients grouped into CP-D (n = 12) or CP-N (n = 8) clusters were analyzed by FACS for the cytokines produced by their CD16^+^ CD11c^+^MHC-II^+^ MDCs in response to activation by TLR3, TLR4, TLR8, and RIG-I agonists. Fig. 1 illustrates the differences in the strengths of the MDC responses between CP clusters using the expression of TNF-α following LPS (TLR4) and 3M-002 (TLR8) stimulations after gating on IL-6^+^.
MDCs for two representative viremics. By gating on IL-6⁺ MDCs, which is itself not inhibited by HCV, before measuring IL-12 and TNF-α production, differences between patients were effectively normalized and resolved at the single-cell level, as previously explained (14). LPS and 3M-002 stimulated high TNF-α expression in MDCs from patient CP-N P55 (Fig. 1A, bottom row). Conversely, patient CP-D P50 demonstrated a loss of TNF-α-secreting effectors specific to LPS, as indicated by the diminished MFI and the percentage of positive cells relative to patient CP-N P55 (Fig. 1A). This also was true 24 wk after entry of patients into the study. Similar results were obtained for all clustered viremics used in this study (Fig. 1B). Importantly, defective cytokine production in the CP-D cluster was not due to general failure to respond to PRR agonists, because CP-D patients maintained intact IL-12 and TNF-α production to MyD88-associated responses (Fig. 1B; see 3M-002). To determine whether the initial CP phenotypes observed were maintained over time in CHC, we tracked the PRR activity longitudinally in 11 of 20 viremics where multiple longitudinal visits during CHC existed (0–52 wk). In CP-D and CP-N patients, the TLR response profiles were stable over weeks (Fig. 1C) and for up to 1 y (data not shown) of CHC. MDCs of CP-D viremics were also unable to induce cytokines in response to transfected full-length HCV genomic RNA (Fig. 1C), which contains pathogen-associated molecular patterns sensed by RIG-I (22), in contrast to CP-N patients who had intact responses to all agonists tested, similarly to aviremics. No known clinical characteristics, including infecting genotype, history of antiviral therapy, PVL, and serum aspartate transaminase and alanine transaminase levels, correlated with or predicted those subjects with preserved TRIF- and IFN-β promoter stimulator-1 (IPS-1)–dependent PRR potentials (CP-N subgroup; Table I). Thus, all viremics preserved or had attenuated PRR potentials over the time window of CHC analyzed, independent of disease progression, but concomitantly with CVL (see later discussion). To investigate further the MDC functional status during CHC, we analyzed the ability of MDCs to respond to PRR signaling by upregulating MHC class II and CD86 and inducing CCR7 expression. The expression of the aforementioned molecules was comparable among MDCs from healthy donors and infected clusters after activation (data not shown). It seems that HCV suppresses distinct facets of MDC function during viral persistence (e.g., IL-12 expression), whereas other facets, such as costimulation, are not affected. Altogether, these data indicate that deregulated PRR responses follow a bimodal distribution among viremics, rather than a general, uniform shift toward lower levels in all patients; are restricted to select PRRs; and are a long-lasting MDC intrinsic defect during CHC restricted to the CP-D subgroup of viremics.

**HCV inhibitors can restore DC-mediated innate sensing in CP-D patients**

Because FACS-purified DCs from CP-Ds have a 10-fold greater CVL than CP-Ns (14), it is plausible that these dysfunctions in PRR responsiveness described above (Fig. 1) result from the ability of HCV to interfere with key signaling adaptors within the TRIF- and IPS-1–coupled PRR pathways. We were not able to isolate MDCs from HCV-infected patients in sufficient numbers to conduct direct experiments to look for virus-induced alterations in these antiviral pathways. Therefore, to test this hypothesis, CP-D samples were treated for 24 h with HCV NS3 protease BILN2061 and NS5B polymerase VIR235 inhibitors prior to monitoring the response to PRR agonists (LPS and 3M-002) of similar stimulatory capacity by FACS. Coincident with a decrease in the proportion of CVL (Fig. 2A, right panel), stimulation with LPS in the presence of inhibitors resulted in a 2-fold increase in the frequency of IL-12⁺TNF-α⁺ MDCs compared with stimulation of vehicle-treated cells in one representative CP-D patient (Fig. 2A, left panel). For 8 of 10 CP-D individuals tested who showed BILN2061 or VIR235-induced reductions in their CVL (Fig. 2B, right panel), incubation of PBMCs with either inhibitor resulted in a significant increase in LPS-activated cytokine-producing MDCs (p = 0.0016; Fig. 2B, left panel). Because quantitative PCR analysis could not be performed directly on purified DCs, it is conceivable that, in some cases, we did not detect significant viral RNA inhibition (<50% inhibition) after 24 h because of the cell-associated HCV RNA decay rate [t₁/₂ ~11 h (23)]; the presence of a large (and variable) fraction of cells types in PBMCs that harbor HCV RNA in vivo but are not expected to support replication (24); and the small number of MDCs nonfunctional for cytokine

Table I. Virological and immunological characteristics of HCV-infected patients

<table>
<thead>
<tr>
<th></th>
<th>Aviremic (n = 16)</th>
<th>CP-N (n = 9/23 [39%])</th>
<th>CP-D (n = 14/23 [61%])</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y; mean ± SD)</td>
<td>50 ± 8</td>
<td>48 ± 4</td>
<td>51 ± 8</td>
<td></td>
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<tr>
<td>Gender (n; females/males)</td>
<td>8/8</td>
<td>4/5</td>
<td>5/9</td>
<td></td>
</tr>
<tr>
<td>Genotype (n)</td>
<td></td>
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<tr>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2</td>
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<tr>
<td>1a</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td></td>
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<tr>
<td>1b</td>
<td>3</td>
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<td>6</td>
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<tr>
<td>2b</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
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<tr>
<td>Unknown</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Alanine transaminase (U/l; mean ± SD)</td>
<td>N/A</td>
<td>103 ± 55</td>
<td>61 ± 43</td>
<td></td>
</tr>
<tr>
<td>Aspartate transaminase (U/l; mean ± SD)</td>
<td>N/A</td>
<td>68 ± 49</td>
<td>52 ± 28</td>
<td></td>
</tr>
<tr>
<td>PVL (log₁₀ IU/ml; log₁₀ ± SE)</td>
<td>&lt;1.7</td>
<td>6.5 ± 0.1</td>
<td>6.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>MFI⁺ expression</td>
<td>5.4 ± 1.1</td>
<td>9.3 ± 1.2*</td>
<td>10.9 ± 1.1**</td>
<td>0.0205* vs. aviremics</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0176** vs. aviremics</td>
</tr>
<tr>
<td>MFI⁺</td>
<td>2037 ± 224</td>
<td>2737 ± 199**</td>
<td>2588 ± 88**</td>
<td>&lt;0.01** vs. aviremics</td>
</tr>
</tbody>
</table>

*Measured with Cobas AmpliPrep/TaqMan HCV Test at study entry (week 0).
Mann-Whitney U rank-sum test, comparing PD-L1⁺ MDC blood frequency.
One-way ANOVA (p = 0.0008) with Tukey posttest, comparing PD-L1 geometric MFI on MDCs (mean ± SEM).
CP-N versus aviremics; **CP-D versus aviremics.
N/A, not available.
production [30 of 1000 overall DCs in blood are defective (14)]. Nevertheless, consistent with the above observations, there was a positive correlation between HCV inhibition (calculated from the residual levels of HCV RNA in PBMCs at 24 h posttreatment) and increases in cytokine-expressing MDC frequencies after LPS stimulation \( (p = 0.0022; \text{Fig. 2C}) \), indicating that impaired PRR functions of MDCs can be restored ex vivo by inhibiting cell-associated HCV RNA abundance. Moreover, in this setting, there was no change in the ability to respond to 3M-002 (Fig. 2D, 2E) or an increase in IL-6–producing frequencies for any agonist studied (Fig. 2F), confirming that HCV suppresses specific PRRs and that it alters select functions of MDCs without affecting all functional properties. As expected, treatment with HCV inhibitors produced no enhancement of LPS- or 3M-002–specific responses in CP-N subjects or uninfected donors (data not shown). Altogether, in CP-D patients, these results demonstrate a direct relationship among HCV, impaired PRR-dependent activation, and attenuated IL-12 and TNF-\( \alpha \) synthesis in a proportion of MDCs producing intact levels of IL-6.

Enhanced HCV-specific CD8\(^+\) T cell frequency and polyfunctionality in viremics with normal MDC functions (CP-N subgroup) despite high PVLs and weak HCV-specific CD4\(^+\) T cell help and PD-1/PD-L1 coinhibitory signals

Given the pivotal role of PRRs and DCs in tuning the adaptive immune response, we investigated whether MDC inactivation by HCV differentially impacts the functional signatures of virus-specific T cells in the context of CHC by analyzing CD8\(^+\) T cell responses to HCV and CMV, EBV, and influenza (CEF) peptides in clustered CP-D \( (n = 12) \), CP-N \( (n = 7) \), and aviremics \( (n = 6) \) by FACs. Limitations imposed by cell numbers allowed only NS3- and NS5B-specific CD8\(^+\) T cell responses to be assessed for HCV; these two nonstructural proteins were selected because they constitute immunodominant targets of HCV-specific immune responses, irrespective of virological outcome (25). Consistent with previous findings reported for viremics and SVRs (<50 HCV RNA IU/ml) (26, 27), the frequencies of HCV-specific CD8 memory (identified by CD107a surface mobilization and IFN-\( \gamma \), TNF-\( \alpha \), and IL-2 production) were low, ranging from 0.01–0.7%, and targeted few regions of the two NS proteins tested (Supplemental Fig. 1, Supplemental Table 1). However, significant differences were found between HCV-specific CD8 frequencies observed in CP-N patients \( (0.34\% \pm 0.09\%, n = 7) \) compared with those of the CP-D cluster \( (0.04\% \pm 0.01\%, n = 12) \); \( p = 0.0013 \) by Mann-Whitney \( U \) test); none of the CP-D viremics showed frequencies that overlapped with those of CP-N subjects \( (71\% \) of whom had \( \geq 0.10\% \) CD8 response per Ag; Supplemental Table 1). The frequencies of CEF-specific CD8\(^+\) memory T cells in peripheral blood were not decreased in any of the groups; they actually were higher in most patients than the responses seen against HCV peptides \( (p = 0.0002 \) by Wilcoxon signed-rank test; Supplemental Table 1). We also explored whether surface phenotypic markers on virus-specific CD8\(^+\) T cells differed depending on patient group. Among HCV-specific CD8\(^+\) T cells, the expression of CD27, CD45RO, and PD-1 was highly heterogeneous and did not differ between groups (data not shown). Together, these

**FIGURE 2.** HCV reversibly suppresses TRIF-dependent MDC activation in CP-D patients. A. Left panels: representative data from 1 of 10 CP-D subjects stimulated with LPS following a 24-h incubation with vehicle alone (V; 0.1% DMSO), protease inhibitor BILN2061 (PI), or polymerase inhibitor VIR235 (Poll). Ten-percent contour plots are gated on IL-6–expressing MDCs. Numbers in the bottom right corner indicate the percentages of cells in designated areas. Right panel: HCV RNA abundance in PBMCs (CVL; black bars) for each condition. Numbers are the percentages of HCV RNA inhibition \( (n = 10) \). Statistical comparisons were made using the Wilcoxon matched-pairs test. Data are averaged values for replicates for one subject from one experiment each. B. Left panel: percentage of IL-12\(^+\)TNF-\( \alpha \)– MDCs of IL-6\(^+\) MDCs in the presence or absence of HCV inhibitors \( (n = 10) \). Statistical comparisons were made using the Wilcoxon matched-pairs test. Data are averaged values for replicates for one subject from one experiment each. Right panel: numbers indicate the percentage of inhibition calculated from the levels of HCV RNA in PBMCs at 24 h in the presence of PI or Poll; the extent of inhibition is highlighted by color code. Relationship between LPS (C) or 3M-002 (D) responsiveness and effect of HCV RNA inhibition by PI or Poll on cytokine production (sum of IL-12– and TNF-\( \alpha \)–expressing MDC frequencies in inhibitor-treated cultures over vehicle control). Data were analyzed using the Spearman correlation \( (n = 10; \) two measurements per patient). E. Pie charts depicting the mean proportion of all individual MDC functional response patterns for all CP-D patients analyzed by FACs. The value beneath each pie represents the percentage of the total response (IL-6 expression) to the respective stimuli.
findings suggest that exhaustion of T cell functions, rather than quantitative and phenotypic differences, account for the decreased capacity of these viremics to restrict HCV replication, as previously reported (28).

One characteristic of T cell exhaustion is the hierarchical loss of functions: the ability to produce IL-2 and convey cytotoxicity are lost early in the process, followed by deficient TNF-α expression, whereas IFN-γ production is the function remaining intact for the longest time. Therefore, we expected HCV-specific CD8+ T cells in viremics to be exhausted (i.e., to express few simultaneous functions), irrespective of DC functionality. Fig. 3A illustrates a typical multifunctional analysis of HCV-specific CD8+ T cell quality from an infected subject (P63). There were substantial frequencies of IFN-γ–producing CD8+ T cells among NS3 pool 5-stimulated PBMCs from this subject (0.056% of the CD8+ memory subset), which were not identified in the negative-control stimulations. A detailed analysis of the HCV-specific CD8+ T cell quality from P63 revealed a heterogeneous functional profile (Fig. 3A, 3B). Of all memory CD8+ T cells, 0.07% responded to NS3 (Fig. 3B, 3C); IFN-γ production (≈79% of responding cells) was the dominant function. Although about two thirds of responding CD8+ T cells produced TNF-α, degranulation as a measure of cytotoxic potential (CD107a+) only accounted for ≈13% of the total response; these CD8 cells equally lacked IL-2 secretion (Fig. 3A, 3B). The HCV-specific CD8+ T cells present in other CP-D patients exhibited a similar functional profile: a blunted polyfunctional response pattern predominantly consisting of cells exhibiting two (2+) or fewer simultaneous functions and dominated by cells that only produced IFN-γ (Fig. 3D). Unexpectedly, the response of CP-N viremics was enhanced in polyfunctionality, such that cells positive for CD107a (Fig. 3D) and three simultaneous functions were detectable similarly to aviremics (Fig. 3E).
Despite, on average, the almost one-log higher HCV-specific frequency in CP-N than in SVR aviremics (Supplemental Fig. 1) and the equally high PVL between CP clusters (medians: 6.47 ± 0.28 versus 6.26 ± 0.50 log_{10} IU/ml for CP-N and CP-D, respectively; \( p > 0.5 \); Supplemental Table 1). CP-N subjects also showed significantly higher proportions of 2+ cells (\( p < 0.05 \)), as did aviremics (\( p < 0.05 \)), compared with CP-D individuals (Fig. 3E). Moreover, although the proportion of IL-2–secreting HCV-specific CD8+ T cells in aviremics (median 12.5%, \( n = 5 \)) was significantly higher compared with that of CP-Ds (0%; \( p < 0.05 \), \( n = 9 \)), there was an overlap in the quantity of IL-2 production between CP-N aviremics (10%, \( n = 7 \)) and aviremics (Fig. 3F). This was not restricted to responses to certain peptide pools, because IL-2–secreting CD8+ T cells from CP-N were detected in NS3 and NS5B responses, nor was it a generalized intrinsic cellular defect, because CD8+ T cells of viremics (CP-D and CP-N alike) were able to produce IL-2 after CEF or staphylococcal enterotoxin B stimulation (data not shown).

During chronic infections, PD-1/PD-L1 and an inadequate CD4 Th cell environment drive polyfunctional CD8+ T cells to exhaustion (20, 29). Ex vivo HCV-specific CD4 responses were generally below the detection threshold of the assay, irrespective of grouping; when detected, there was no significant difference between CP clusters (\( p > 0.5 \), Mann-Whitney U test; Supplemental Fig. 1, Supplemental Table 1). PD-L1 expression was readily apparent on MDCs (as well as monocytes, Fig. 4A), irrespective of CP grouping (9.3% ± 1.2% versus 10.9% ± 1.1%, \( p > 0.05 \) for CP-N and CP-D, respectively; Table 1), and it was significantly higher than in aviremics (5.4% ± 1.1%, \( p = 0.0025 \) and \( p = 0.0176 \) versus CP-N and CP-D, respectively; Table 1). In addition, PD-L1–positive MDCs from viremics, regardless of CP cluster, exhibited increased levels of surface expression (measured as MFI) of this inhibitory ligand throughout the time period of CHC analyzed compared with aviremics (Fig. 4B). PD-L1 blockade (Fig. 5) increased the number of proliferating HCV-specific CD8+ T cells (measured as the sum of cytokine-producing CD107a+CFSE\textsuperscript{dim}CD8+ T cells over isotype control; \( p = 0.0004 \), Fig. 5A, 5C) in six CP-Ds and four CP-Ns. However, this is without inducing a significant shift in the pattern of simultaneous functions expressed by the responding cells in either CP group after restimulation with the cognate Ag at the end of the period of intraversion in the muldivity assay (Fig. 5B, 5D).

**FIGURE 4.** PD-L1 is upregulated at the surface of MDCs and monocytes in viremics. A. Curves are gated on MDCs (top panels) or CD16+CD14+ monocytes (bottom panels). Numbers on the right of and above bracketed lines indicate the geometric MFI and the percentages of PD-L1–expressing cells in the designated area, respectively. B. Baseline PD-L1 surface expression (geometric MFI) on MDCs from viremics (\( n = 13 \), triangles), aviremics (\( n = 12 \), squares), and HCV-seronegative healthy controls (\( n = 10 \), circles) during 1 y of CHC without treatment. Horizontal bars indicate mean geometric MFI; horizontal line indicates a geometric MFI expression of PD-L1 on these MDCs that is twice the SD of the mean of the geometric MFI of MDCs expressing PD-L1 in controls. Each symbol corresponds to one subject at one time point during CHC. The Tukey one-way ANOVA posttest was used for comparisons between groups and time points. \( *p < 0.05 \); \( **p < 0.01 \); and \( ***p < 0.001 \); \( p = 0.002 \), Mann-Whitney U test between week 0 and aviremics.**

**Discussion**

Functional exhaustion of an initially vigorous polyfunctional T cell response predicts the likelihood of viral persistence for HCV or HIV (20, 29, 30). It is generally viewed that the effects of such pathogens on T cell exhaustion are related largely to repeated Ag stimulation and the lack of CD4 help and PD-1/PD-L1 (or other) coinhibitory signals; however, their nonredundancy (31) implies that all of the factors involved in exhaustion are not defined. An emerging theme in innate immunity postulates that DCs play a critical role in sensing viruses directly through their PRRs and in integrating this information to regulate the quality of the adaptive immune response (32). If generally true, could not functional pathogen recognition by DCs result in least exhausted T cell effector functions in settings of chronic infections, especially because these pathogens have evolved means to inhibit DC function (19)? In this paper, we provide evidence for such a scenario: HCV impairs the activation of DCs via select PRRs by reversibly...
interfering with TRIF- and IPS-1–dependent signal processing during chronic infection; this in turn, associates to exhaustion of polyfunctionality of HCV-specific CD8+ T cells (i.e., loss of IL-2 secretion and CD107a). In unpublished experiments, we found that HCV inhibitor treatment of PBMCs depleted of monocytes, B cells, and plasmacytoid DCs, to retain only MDCs as the APCs, did not alter the polyfunctional CD8+ T cell profiles detected in CP-D patients. These findings indicate that, in our experimental setup, the polyfunctional T cell readout is not affected by the in vitro status of MDC function; therefore, the data presented in this paper reflect an in vivo association. We think that this effect was underappreciated in previous experiments that analyzed CD8 exhaustion directly in virally infected hosts (murine or human), possibly because the infectious agents studied impair multiple functions of DCs, making it difficult to assess the effect that DCs have on the T cell response under chronic viral conditions. Virulence factors that accelerate the viral set-point in a host probably dictate how quickly coinhibitory/immune-suppressive conditioning of Ag-specific T cells is established during acute infection (33), which may lead to release from early immune selection pressure, obviating any advantage of HCV variants with altered cellular tropism to be retained within nonhepatocyte cells. The stochastic nature of viral quasispecies evolution that generates NS3/4A proteases with different catalytic efficiencies (34) may also be involved in the variability in DC function between patients once persistence is established (see later discussion). However, this is a separate issue because the aim of the current study was to determine which T cell functions are disrupted in the presence of interfering with TRIF- and IPS-1–dependent signal processing during chronic infection; this in turn, associates to exhaustion of polyfunctionality of HCV-specific CD8+ T cells (i.e., loss of IL-2 secretion and CD107a). In unpublished experiments, we found that HCV inhibitor treatment of PBMCs depleted of monocytes, B cells, and plasmacytoid DCs, to retain only MDCs as the APCs, did not alter the polyfunctional CD8+ T cell profiles detected in CP-D patients. These findings indicate that, in our experimental setup, the polyfunctional T cell readout is not affected by the in vitro status of MDC function; therefore, the data presented in this paper reflect an in vivo association. We think that this effect was underappreciated in previous experiments that analyzed CD8 exhaustion directly in virally infected hosts (murine or human), possibly because the infectious agents studied impair multiple functions of DCs, making it difficult to assess the effect that DCs have on the T cell response under chronic viral conditions. Virulence factors that accelerate the viral set-point in a host probably dictate how quickly coinhibitory/immune-suppressive conditioning of Ag-specific T cells is established during acute infection (33), which may lead to release from early immune selection pressure, obviating any advantage of HCV variants with altered cellular tropism to be retained within nonhepatocyte cells. The stochastic nature of viral quasispecies evolution that generates NS3/4A proteases with different catalytic efficiencies (34) may also be involved in the variability in DC function between patients once persistence is established (see later discussion). However, this is a separate issue because the aim of the current study was to determine which T cell functions are disrupted in the presence of...

**FIGURE 5.** Blockade of the PD-1/PD-L1 pathway increases the expansion of HCV-specific CD8+ T cells but not their polyfunctionality. PBMCs from patients with detectable HCV-specific CD8+ T cell responses were stimulated with cognate peptides in the presence or absence of PD-L1 blocking Ab for 7 d and analyzed for CFSE dilution and polyfunctionality (measured as in Fig. 3). A and B, Representative data from two viremics following a 7-d incubation with peptides and isotype control Abs or anti–PD-L1 Abs, followed by restimulation with peptides and CD28/CD49d on day 7 (27% density plots). A, Values indicate the percentage of CD3+CD4+CD8+CFSEdim IFN-γ–producing cells out of the HCV-specific total response (shown as italic number). B, Numbers in top right corners indicate the percentages of cells in the designated areas. C, Statistical analysis of data obtained as described in A (Wilcoxon matched-pairs test) for 10 viremics (four with a CP-N phenotype and six with a CP-D phenotype). PD-L1 proliferation index and ratio of total cytokine-producing CD107a+CFSEdim/CD8+ T cells (frequencies in 7-d stimulated cultures) in the presence of anti–PD-L1 blocking Ab over isotype control. Each symbol corresponds to one subject responding to one Ag specificity (NS3 or NS5B, circles; CEF, triangles); 20 and 10 measurements for HCV and CEF, respectively. Differences of less than the upper limit of the 95% confidence interval (dashed line) were considered not significant. D, Pie charts depicting CD8+ T cell polyfunctionality after restimulation with HCV peptides at the end of the period of in vitro expansion in a 7-d assay in the presence of anti–PD-L1 blocking or isotype Ab. Values in the center of each pie represent the total HCV-specific CD8 memory response (median percentage ± SE). The results shown were generated from the determinations in four CP-N and six CP-D responders. E, Fold expansion of HCV- or CEF-specific CD8+ T cells (calculated as in B) corrected for CP cluster grouping.
 impaired DCs, independently of other confounding factors during the chronic phase of HCV infection. In addition, this study demonstrated that unrelated CD8 specificities (i.e., CMV, EBV, and influenza) are functionally normal in CHC, regardless of DC functional differences between viremics, in accordance with the narrow range of immune disorders documented in such patients (3). Thus, the degree to which DCs contribute to T cell exhaustion may be dependent on the type of pathogen and the extent to which it elicits altered innate DC responses in infected individuals. Taken together, the data refute the hypothesis that impairment of PRR-dependent activation of DCs by HCV would invariably lead to erosion of unrelated T cell specificities along with those against HCV. They also suggest that DC inhibition represents a factor that, in synergy with other well-accepted causes (see later discussion), may favor Ag-specific exhaustion of CD8+ T cell polyfunctionality and prolonged host–parasite coexistence, without necessarily causing widespread immune suppression.

Investigation of the possible mechanistic links between HCV and the agonist-specific attenuation of PRR pathways in MDCs revealed that small-molecule HCV inhibitors could restore signaling to TLR4 agonists coincident with reductions in CVL (Fig. 2). These results, together with the conserved responses to TLR8 (known to signal solely through MyD88 (17)), indicate that the muted innate response of DCs does not result from an inability of the initial upstream receptors to detect HCV or other PRR agonist; it results from virus-altered propagation of activation signals along signaling pathways downstream of receptor binding. The addition of an inhibitor, such as BILN2061, would be expected to overcome HCV-dependent attenuation of PRRs (12, 13) in the affected MDCs, reverse this phenotype, and enable greater than normal cytokine induction, because we frequently observed this in treated CP-Ds, but only when TRIF- and IPS-1–coupled signaling pathways were initially intact and specifically suppressed by HCV. Presumably, the defect would limit activation—after TLR3, TLR4, or RIG-I stimulation—of only those APCs that have come into direct contact with HCV. Although sensitivity to BILN2061 treatment does not equate to TRIF cleavage and degradation by NS3/4A (11), our data support the existence of NS3-dependent mechanisms that target TRIF itself or components of the signaling pathway downstream of TRIF in a numerically restrained proportion of MDCs, because RIG-I signaling is equally perturbed, MyD88-dependent activation seems to be intact, and not all CP-D MDCs are unable to respond through the affected PRRs (Fig. 1A), as previously reported (14). However, this does not exclude the possibility that NS3/4A protease-independent mechanisms are also involved, because CP-D patients were equally sensitive to the polymerase inhibitor.

Multiple nonoverlapping factors seem to affect T cell exhaustion (35). Unexpectedly, we found that enhancement in polyfunctionality segregated with the presence of functionally intact DCs (Figs. 3, 5), despite the comparable presence of PD-1 coinhibitory signals and high Ag burden, both of which are presumed to be interdependent causative factors. This is consistent with evidence that PD-L1 negatively impacts the frequency of antiviral T cells in chronic viral infections and can be manipulated to improve CD8 numbers but not all effector functions (36). We acknowledge that, by necessity, the data herein are correlative in nature, making it difficult to exclude that DC and CD8+ T cell dysfunction may be due to separate mechanisms of suppression of both of these responses, unrelated to each other. Because of differences in the HCV-specific CD8 frequencies between CP-Ds and CP-Ns, we should not infer from these results that polyfunctional T cells are necessarily absent in CP-Ds, because they might just fall below the detection threshold of the FACS assay. However, the finding that cells positive for IL-2, CD107a, and three simultaneous functions are detectable in SVRs, similarly to CP-Ns (Fig. 3), despite the average almost one-log higher HCV-specific CD8+ T cell frequencies in the latter group, excludes the possibility that the increased number of polyfunctional cells in CP-Ns is largely a function of the much higher HCV frequencies in these subjects. Alternatively, a more pronounced loss of CD4+ T cell help in CP-Ds might be hypothesized to be the issue (2, 3). However, in the few patients in whom we detected HCV-specific CD4 responses, the frequencies per Ag were very low and did not differ between clusters (Supplemental Fig. 1, Supplemental Table 1). Although the requirement for CD4+ T cell help throughout chronic infection to sustain CD8+ T cell function (37, 38) is not in dispute, PRR-activated DCs could represent a backup mechanism for situations in which CD4 responses are defective. This would be in keeping with findings by other investigators in which the adoptive transfer of autologous DCs loaded in vitro with whole-inactivated HIV-1 or therapeutic vaccination with a recombinant viral vector, both of which recruit functional DCs into the APC pool, induces protective antiviral immunity and viral suppression in the setting of chronic HIV-1 infection (39) and synergizes with PD-L1 neutralization to clear infection in CD4+ T cell help-deficient LCMV clone 13-infected mice (40), respectively. The addition of TLR3 or TLR9 agonists to cationic liposomes can elicit in vivo activation of CD8+ T cells, independent of CD4+ T cell help (41). Accordingly, Johnson et al. (42) showed that PRR-mediated CD40L upregulation on DCs promotes functional CD8+ T cell activation in the absence of CD4 help, providing a mechanism by which pathogens may elicit (and sustain) helper-independent CTL immunity through functional virus-presenting DCs during chronic infection. Alternatively, Schmitt et al. (43) showed that IL-12 from PRR-stimulated DCs directly acts on memory CD4+ T cells to promote the secretion of IL-21. It is plausible that differences in the abundance of IL-21–dependent signals produced by CD4+ T cells are involved in the increased exhaustion of polyfunctional CD8+ T cells (37, 38) in CP-Ds relative to CP-Ns; this awaits formal examination.

Our hypothesis that PRRs on DCs are implicated in sustaining polyfunctional responses, despite chronic infection, does not discredit a role for PD-1 [and high Ag levels (44)]. CD8+ T cells only eliminate viruses after colocalization with cells that are already infected and, thus, cell-cycle deregulation by coinhibitory receptors is likely to be the mechanism accounting for the loss of immunologic control once viral persistence is established, even in patients with moderately improved T cell polyfunctionality. A component of PD-1 expression is regulated by increasing antigenemia; following the initiation of antivirals, PD-1 and PD-L1 expression decreases on HCV- and HIV-specific CD8+ T cells and MDCs, respectively (21, 45, 46). It is possible to block signaling through individual coinhibitory receptors, and the potential benefits of this strategy in controlling chronic viral infections have been discussed recently (7). By ameliorating PD-L1–mediated inhibition in chronic infections, T cells are able to expand, thereby re-establishing high frequencies of existing polyfunctional virus-specific CD8+ T cells that can exert effective control over infection (35); this may not prove as beneficial when innate immune functions of DCs are impaired (40). In fact, in ongoing experiments we find that HCV-infected patients with a CP-N status have markedly improved viral control and enhanced SVR rates compared with CP-D patients on pegylated IFN-α and ribavirin therapy (I.G. Rodrigue-Gervais, H. Rigsby, B. Willems, and D. Lamarre, manuscript in preparation). How much more effective might interventions be if they were also taken to a level upstream of coinhibitory regulation of T cell function: at the level of those yet undefined pathways in DCs that are connected to preserved IL-2
secretion and CD107a mobilization by CD8+ T cells, despite chronic infection? Focused studies in this direction have the potential to provide the vaccine field with novel DC-based therapeutics to recover T cell polymorphism to tip the balance to immune control and clearance of virus once immune-suppressive signals are removed (e.g., PD-1) (29).

In summary, the presence of functioning PRRs on DCs in CHC is associated with less virus-specific exhaustion of CD8+ T cell polymorphism despite high Ag loads and lack of adequate CD4 help and PD-1/PD-L1 inhibitory signals. We propose that Ag persistence may not intrinsically be a detrimental factor for all T cell functions per se. Rather, the presence of virus-presenting DCs with intact innate functions during CHC could mitigate the impact of immune-suppressive signals on T cells, thus helping to support the maintenance of a continuously activated CD8+ T cell pool that does not necessarily exhaust or lose function and yet, because of its small size, is limited in its ability to control infection. Further elucidation of the triggered changes in DCs by which PRRs impact exhaustion is expected to offer insights into how to maintain antiviral immunity to chronic pathogens.

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