HCV core and NS3 proteins manipulate human blood-derived dendritic cell development and promote T\(_h\) 17 differentiation

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Abstract

Hepatitis C virus (HCV) chronic infection is characterized by low-level or undetectable cellular immune response against HCV antigens. HCV proteins affect various intracellular events and modulate immune responses, although the mechanisms that mediate these effects are not fully understood. In this study, we examined the effect of HCV proteins on the differentiation of human peripheral blood monocytes to dendritic cells (DCs). The HCV core (HCVc) and non-structural 3 (NS3) proteins inhibited the expression of CD1a, CD1b and DC-SIGN during monocyte differentiation to DCs, while increasing some markers characteristic of macrophages (CD14 and HLA-DR) and also PD-L1 expression. Meanwhile, HCVc and NS3 could induce differentiating monocytes to secrete IL-10. However, anti-IL-10 mAb could not reverse HCVc and NS3 inhibition of monocyte differentiation into DCs. The HCVc and NS3 proteins increased IL-6 secretion both in immature and in fully differentiated DCs and also promoted CD4\(^+\) T-cell IL-17 production. Since T\(_h\) 17 cells are active in many examples of immunopathology, these effects may contribute to HCV autoimmune responses in chronically infected patients.

Keywords: antiviral responses, immune subversion, innate immunity, programmed death ligand, toll-like receptor

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease worldwide. An estimated 4 million people are infected with HCV in the USA and 170 million are infected worldwide. Hepatitis C poses a major risk for the development of liver cirrhosis and hepatocellular carcinoma. In the early phase of acute infection, HCV continues to replicate in the liver, overcoming innate and acquired immunity in a majority of infected humans. Sustained, vigorous and multiepitope-specific CD4\(^+\) and CD8\(^+\) T-cell responses are essential for spontaneous HCV clearance (1). The virus modulates the host's immune system (2, 3), and the mechanism of the immune imbalance that favors HCV persistence is yet to be fully understood.

Dendritic cells (DCs) are important for the initiation of T-cell responses to many foreign antigens. The antigen uptake and presentation capacities of DCs enable them to prime and activate naive T cells. Immature DCs are optimally phagocytic and they also pinocytose protein antigens. However, DCs must be fully differentiated before serving as efficient antigen-presenting cells. The DCs isolated from patients chronically infected with HCV display a reduced capacity to induce T-lymphocyte activation (4–9). These monocyte-derived DCs also displayed a severely suppressed capacity to produce the T\(_h\)1-inducing cytokine IL-12 in response to TLR or CD40 ligand (CD40L) stimulation (4, 8, 10). However, this conclusion remains controversial since other studies found that monocyte-derived DCs from chronically infected patients could undergo full phenotypic and functional maturation (11, 12). Further investigation is needed to clarify whether DCs are indeed disabled in the setting of chronic hepatitis C, and furthermore, whether this subversion of monocyte differentiation contributes to the autoimmune response.
HCV proteins and DC differentiation

development of HCV persistence or is simply a consequence of active HCV infection. The HCV core (HCVc) and NS3 proteins can trigger inflammatory cell activation via the pattern-recognition receptor TLR2 and induce multiple inflammatory cytokines but fail to activate macrophages from TLR2 or MyD88-deficient mice (13). The HCVc and NS3 proteins interact with many host proteins (14,15); however, the mechanisms by which these proteins modulate the innate immune system and contribute to disease are not well understood.

Because of the aforementioned role of TLR2 by HCV protein stimulation of macrophages in secreting inflammatory cytokines (13) and our previous studies pointing to the subversion of the liver macrophage antiviral response to cytokines (13) and our previous studies pointing to the mechanisms by which these proteins modulate the immune system and contribute to disease are not well understood.

Detection of NF-κB1 (p50) mRNA

NF-κB1 (p50) mRNA was quantified using real-time reverse transcription–PCR (TaqMan). Total RNA was isolated from differentiating DCs using RNAeasy Micro Kit (Qiagen, Valencia, CA, USA) and the cDNA was synthesized using oligo(dT) primers (AffinityScript QPCR cDNA Synthesis Kit; Stratagene/Agilent Technologies, CA, USA). Real-time detection was carried out with Mx3005P instrument (Stratagene, La Jolla, CA, USA) using a master mix (TaqMan® Universal Master Mix, No Amp Erase® UNG) from Applied Biosystems (Foster City, CA, USA) and primers and probes designed with RealTime-Design™ software and synthesized by Biosearch Technologies (Novato, CA, USA). The amount of human NF-κB1 (p50) mRNA (NM_003998) was detected using the probe FAM-5’- TGAAGTGATCCAGGCAGCCTCC-GAAGCAAT-3’ (primer 1) and 5’-GGGCCTG AGAGGTTGCTTCT-3’ (primer 2) and the signal was normalized to the expression of EF-1a (EEF1A1, NM_001402) which was detected with Cal Fluor Orange560-5’-ACCACCGAGAAGGAAAT-3’ (primer 2) and primers 5’-GGTGTGGACTGTGGTCTCA-BHQ1-3’ (probe) and primers 5’-TGTT GGTATTTGACTGTGT-3’ (primer 1) and 5’-TGACTGGAG-CAAAGTGT-3’ (primer 2). Samples were analyzed in duplicate and no template controls were included for each master mix. Relative quantification of expression (ΔΔCt method, ABIUser-Bulletin#2; http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf) was used to calculate the gene expression data.

Methods

Buffy coat

These studies were approved by the IRB of the University of Rochester Medical Center. We used buffy coats provided by New York Blood Center, and informed consent was provided according to protocols of the New York Blood Center.

HCV-encoded proteins, cytokines and reagents

Recombinant HCVc (aa 2–192 of the HCV polyprotein), NS3 (aa 1450–1643 of the polyprotein) and β-galactosidase expressed and purified identically to HCVc and NS3 proteins were purchased from Biodesign (Saco, ME, USA); recombinant HCV E2 proteins were purchased from Immuno-Diagnostics, Inc. (Woburn, MA, USA); human recombinant (hr) granulocyte macrophage-stimulating factor (GM-CSF), IL-4 and tumor necrosis factor (TNF)-α were obtained from R&D Systems (Minneapolis, MN, USA). Stock solutions of GM-CSF, IL-4 and TNF-α were prepared in the manufacturer’s buffer and then diluted in RPMI-1640 serum-free medium; aliquots were kept at –80°C.

Limulus amoebocyte lysate assay for detection of LPS contamination

The QCL-1000® chromogenic LAL endpoint assay (Cambrex, Cottonwood, AZ, USA) was used to detect endotoxin contamination of HCVc and NS3 proteins following the manufacturer’s protocol. Briefly, protein-free, phenol–water extracted ultrapure Escherichia coli LPS was used to create a linear standard curve from 1 to 100 pg ml⁻¹. HCVc and NS3 were serially diluted to determine a concentration of agonist within the standard curve of the assay. The HCVc and NS3 preparations were determined to have <1 pg ml⁻¹ LPS contamination.

Isolation of monocytes from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Paque (WWR International) density gradient centrifugation of blood obtained from buffy coats of healthy volunteers from the blood bank of New York City Blood Center. For magnetic cell sorting, a monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for the purification of monocytes without ligation of any of their surface molecules. The purity of the CD14⁺ T cells determined by flow cytometry was always in the range of 90–95%.

Preparation of DCs from monocytes

Cultures of DCs were generated from PBMCs as described previously (18). In brief, the purified monocytes were differentiated into DCs by culture in RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 IU ml⁻¹ penicillin and streptomycin, supplemented with 400 IU ml⁻¹ GM-CSF and 400 IU ml⁻¹ IL-4 at 37°C with 5% CO₂ in the presence or absence of HCV-encoded proteins for 5 days. Fresh medium and cytokines were added at days 2 and 4 of culture. To generate fully differentiated DCs, 200 IU ml⁻¹ of TNF-α was added for the final 48 h of culture.
For studying the effect of HCV proteins on the development of DCs, a serial dose of HCV proteins was added to separate cultures of monocytes on days 0, 1, 2, 3 and 4. After the addition of HCV proteins, all cultures continued for total 5 days. The cells were harvested and studied for the development of DCs by flow cytometry.

Preparation of naive CD4+ T cells
Naive CD4+ T cells were isolated from PBMC depleted of CD14+ cells. Cells were treated with anti-CD45RO antibody-conjugated magnetic microbeads (Miltenyi Biotec). After absorbing CD45RO+ cells to a negative selection column (MACS 25 LD; Miltenyi Biotec), CD45RO+ cells in the pass through fractions were treated with anti-CD4 antibody-conjugated magnetic microbeads (Miltenyi Biotec) and the resulting CD4+CD45RO+ cells were collected by positive selection. The naive CD4 T-cell fraction (CD4+CD45RA+ cells, >90%) was then used for these experiments.

T-cell proliferation assays
Peripheral blood monocytes were induced by GM-CSF and IL-4 to differentiate into DCs in the presence or absence of HCV-encoded proteins, the immature DC were washed twice and co-cultured with allogeneic PBMCs, which were stained with 5 μM CFSE (Molecular Probes) according to the manufacturer’s recommended protocol, in RPMI supplemented with 5% AB serum (1:20 DC/PBMC ratio). After 7 days, when clumps were visible, cells were collected and double-stained with PE Cy5-conjugated anti-CD3 and PE-conjugated anti-CD4 (BD Biosciences). To further characterize the proliferating lymphocyte subpopulations, CD4+ T cells were purified by negative selection from allogeneic normal healthy donors using a CD4+ T-cell Isolation Kit II (Miltenyi Biotec). CFSE-labeled allogeneic CD4+ T cells were co-cultured with DCs (1:20 DC/CD4+ ratio) for 7 days, cells were harvested, fixed in 1% formaldehyde and analyzed by flow cytometry.

Flow cytometry
Staining of cells and analysis on a flow cytometer (FACSScan; BD Biosciences) were done as described (19, 20). Cell surface staining was performed using the following anti-human mAb: anti-CD1a-FITC, anti-CD1b-FITC, anti-CD1c-FITC, anti-CD14-FITC, anti-DC-SIGN-FITC, anti-CD206-FITC, anti-CD80-FITC, anti-CD83-FITC, anti-CD86-FITC and anti-HLA-DR-PE, which were obtained from BD Biosciences. Anti-PD-L1 was obtained from eBioscience. Intracellular staining was performed using the following anti-human mAb: TLR2, IFN-γ, IL-4 and IL-17, obtained from eBioscience. The data acquired were analyzed with FlowJo (Treestar software).

Analysis of cytokines
Cytokine concentrations were measured using a multiplexed cytokine bead assay as previously described (16, 20). Aliquots of 100 μl supernatant were sampled at different time points from cultures of DCs.

Statistical analysis
The significance of experimental differences was analyzed using the paired non-parametric Wilcoxon test, administered via Prism software.

Results
Effect of HCV proteins on the development of immature DCs
To determine whether HCVc and NS3 modify the development of DCs, monocytes were cultured in cRPMI medium with increasing concentrations of HCVc and NS3 in the presence of GM-CSF and IL-4 for 5 days. We consistently found that HCVc and NS3 caused the down-regulation of CD1a and CD1b but up-regulation of HLA-DR and PD-L1 (Fig. 1A). The results from seven separate experiments are shown in Fig. 1B. The expression of CD1a and CD1b was decreased in proportion to HCVc and NS3 concentrations. In contrast, HLA-DR and PD-L1 expressions were increased (Fig. 1C).

Duration of culture required for the development of immature DCs in the presence of HCV proteins
To determine the duration of DC development from monocytes, the monocytes were cultured as a function of incubation duration in the presence of HCVc and NS3 (10 μg ml−1) in cRPMI containing GM-CSF and IL-4. The recombinant proteins were added on day 0 of the culture. We found that the expression of CD1a, CD1b, CD1c and DC-SIGN was up-regulated as culture time increased, but HCVc and NS3 treatment delayed the loss of CD14 (HCVc and NS3 versus control: day 1 and 2, P < 0.01; day 1 and 2, CD1c at day 2 and DC-SIGN at day 5, P < 0.05); almost all monocytes became CD14 negative after culture for 2 days, but HCVc and NS3 treatment delayed the loss of CD14 (HCVc and NS3 versus control: day 1 and day 2, P < 0.01). The PD-L1 expression was biphasic: at day 1, PD-L1 was highly up-regulated (day 1 versus day 0: P < 0.01), while at day 2, PD-L1 expression decreased again. The mechanism for the biphasic nature of PD-L1 expression was not elucidated, but PD-L1 expression was still much higher in the presence of HCVc and NS3 than in the control (HCVc and NS3 versus control: days 1, 2 and 5, P < 0.01) (Fig. 2).

sTLR2 blocks the effect of HCV proteins after initiation of cultures for monocyte-derived DCs
The level of TLR2 expression was examined on differentiating DCs in the presence or absence of HCV-encoded proteins, and this showed that the differentiating cells expressed TLR2 at a higher level on day 1 than at any other time point (day 1 versus day 0: P < 0.01; day 1 versus days 3 and 5: P < 0.05; Fig. 3A). To investigate if the induction of TLR2 gene expression could also be seen at the level of transcription factor induction, we measured the level of NF-kB1 (p50) mRNA (Fig. 3B and C) and observed that in the presence of HCV proteins higher levels of NF-kB1 expression is induced. However, only the effect of HCVc
protein but not the NS3 protein was found to be statistically significant (HCVc versus medium: $P < 0.05$). Consequently, we investigated whether sTLR2 could block the effect of HCV proteins after initiation of cultures for monocyte-derived DCs. HCVc and NS3 pretreatment with sTLR2 (range from 0 to 10 $\mu$g ml$^{-1}$) added to the initiation of monocyte cultures on day 1 caused the dose-dependent recovery of CD1a and CD1b expression on immature DCs in comparison with cultures free of HCV proteins ($P < 0.05$ for all pairwise comparisons of HCVc and NS3 alone versus HCVc and

Fig. 1. Effect of HCV proteins on the development of immature DCs. Purified monocytes were cultured in RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 IU ml$^{-1}$ penicillin and streptomycin, supplemented with 400 IU ml$^{-1}$ GM-CSF and 400 IU ml$^{-1}$ IL-4 without HCV protein or with β-galactosidase, HCV E2, HCVc and HCV NS3. CD1a, CD1b, HLA-DR and PD-L1 expressions were studied by flow cytometry after 5 days culture (A). Mean fluorescence intensity (MFI) of mean ± SD from seven separate experiments are shown as (B), MFIs of CD1a and CD1b are significantly down-regulated in the presence of NS3 and HCVc comparing with controls (β-galactosidase, HCV E2 and medium) (CD1a: **$P < 0.01$; CD1b: *$P < 0.05$); MFIs of HLA-DR and PD-L1 are significantly up-regulated in the presence of NS3 and HCVc (HLA-DR: *$P < 0.05$; PD-L1: **$P < 0.01$). (C) Effect of HCV and NS3 protein concentration on the development of immature DCs. Shown is one of five representative experiments.
NS3 plus sTLR2; Fig. 3D). We interpret this to be a result of the sTLR2 forming a stable complex with HCVc or NS3 proteins, thus competitively blocking these proteins from an effective interaction with cell surface TLR2. While there was no specific negative control protein for sTLR2 in these experiments, we allowed the GM-CSF/IL-4, GM-CSF/IL-4/sTLR2 and GM-CSF/IL-4/HCVc (NS3) cultures to act as the negative control.

HCV proteins result in monocyte-derived DCs impaired CD4+ T-cell expansion

In the light of the phenotypic features exhibited by HCV proteins-induced DCs, we then analyzed the capacity of these cells to stimulate the proliferation of allogeneic T cells in mixed leukocyte reactions for assessing antigen-presenting (in this case allogeneic antigens) function of DC. The percentage of CD4+ T-cell proliferation of mean ± SD is shown in Fig. 4; we found that HCVc and NS3 inhibit DC-induced allogeneic CD4+ T-cell expansion in both PBMCs and pure CD4+ cells co-cultured with DCs (PBMC: HCVc and NS3 versus medium, P < 0.05; pure CD4+ cells: HCVc versus medium, P < 0.01; NS3 versus medium, P < 0.05).

The significance of IL-10 in HCV effects on DC development

The HCVc and NS3 proteins engage TLR2 on peripheral blood monocytes and induce multiple inflammatory cytokines, but they inhibit IFN-α production by plasmacytoid dendritic cells, and this action depends on TLR2-mediated IL-10 production (21). In addition, IL-10 was reported to be involved in the DCs’ differentiation (22). In the present study, the secretion of IL-10 by HCVc and NS3-treated DCs was analyzed. The results from one of the three separate experiments are shown in Fig. 5. We consistently found that HCVc and NS3 could induce monocyte IL-10 secretion at day 1, and this continued but at greatly diminished level by day 2 and was undetectable by day 5. Treatment with GM-CSF and IL-4 accelerated the disappearance of IL-10 (Fig. 5A and B). We examined whether the IL-10 could be responsible for the effect of HCV proteins on monocyte differentiation to DCs. We found that the addition of anti-IL-10 mAb could not reverse the...
impact of HCV proteins on monocyte differentiation (\(P > 0.05\) for all pairwise comparisons of HCVc and NS3 alone versus HCVc and NS3 plus anti-IL-10 mAb; Fig. 5C), leading us to conclude that the viral proteins’ stimulation of IL-10 had no relevance to their impact on the differentiation toward DCs.

HCVc increased DC IL-6 secretion
In order to evaluate whether HCVc protein affects the cytokine profiles of DCs, GM-CSF and IL-4 were used to induce monocyte differentiation to DCs for 5 days in the absence or presence of HCVc, and the supernatants were analyzed for cytokine content. We found that HCVc increased significantly the secretion of IL-6 by immature and mature DCs (Table 1). To further analyze these responses, we also determined the cytokine profile of immature and fully differentiated DCs in the presence of a serial dilution of HCVc at a range of concentrations and showed that HCVc could increase IL-6 secretion from both immature and fully differentiated DCs (Fig. 6A).

HCV proteins facilitate capacity of DC to induce IL-17 production from naive CD4 T cells via IL-6
We tested the capacity of DCs to induce IL-17 production by purified CD4+ T cells. The CD4+ T cells were cultured with fully differentiated DCs, pretreated with concentrations ranging from 0 to 20 \(\mu\)g ml\(^{-1}\) of the two HCV proteins for 6 days. The cells were re-stimulated with phorbol myristate acetate and ionomycin, followed by brefeldin A for 5 h. Intracellular IFN-\(\gamma\) and IL-17 staining were used to analyze the CD4+ T cells’ polarization. The data in Fig. 6(B and C) show that HCVc and NS3-pretreated DCs could induce CD4+ T-cell IL-17 production as well as increase IFN-\(\gamma\) secretion. Since HCVc could increase IL-6 secretion from both immature and fully differentiated DCs, we hypothesize that IL-6 may play a role in HCVc and NS3-pretreated DCs inducing CD4+ T-cell IL-17 production. The result is shown in Fig. 6(D and E) that extraneous IL-6 addition increase DC-induced CD4+ T-cell IL-17 expression. In contrast, IL-6 neutralization decreases HCVc and NS3-pretreated DCs-induced CD4+ T-cell IL-17 expression.
**Fig. 4.** Induction of allogeneic lymphocyte expansion by DC. Immature DCs were generated as above in the presence or absence of HCV-encoded proteins, collected at day 5 of culture, were washed and seeded in mixed cultures with CFSE-labeled allogeneic PBMCs (A) or CD4+ T cells (B) at 1:20 ratios. At day 7, cells were double-stained with anti-CD3 and -CD4, as described in the Methods. The CFSE versus CD4 shows the gated CD3+ lymphocyte population. Quadrants were set on the basis of unstimulated and CFSE-labeled cells stained with isotype-control antibody, and the percentage of CD4+ T-cells proliferation of mean ± SD is showed. The data were statistically significant (P < 0.05) for HCVc and NS3-treated DCs-induced CD4+ T-cells proliferation when compared with control (medium) in both co-cultured with allogeneic PBMC and pure CD4+ T cells. Representative experiments from one donor out of three are reported in (A) and (B).

**Discussion**

In this report, we document that HCV proteins modify the differentiation of peripheral monocytes into mature DCs. These experiments were undertaken in a two-step stimulation model. First, monocytes were induced to differentiate to immature DCs in the presence of HCV proteins; second, these DCs were further induced to full functional maturation by TNF-α stimulation. The data reveal that the two HCV-encoded proteins interfere with the process of DCs maturation. In a series of experiments, we consistently found that during the in vitro differentiation of monocytes into DCs, the presence of either HCVc or NS3 caused down-regulation of CD1a and CD1b but up-regulation of HLA-DR and PD-L1 (Fig. 1A and B), and these effects were HCV protein dose-dependent (Fig. 1C).

This observation suggests that the HCV-encoded proteins could be engaged in a form of immune subversion, impairing the normal differentiation of DCs. To test this idea further, we investigated the effect of HCV proteins during the differentiation of DCs from human peripheral blood monocytes and discovered that, in addition to CD1a, CD1b, HLA-DR and PD-L1, the expression of CD1c, CD14 and DC-SIGN was also affected by HCVc and NS3 during monocyte differentiation to DCs (Fig. 2). We also found that HCV proteins had to be included in the culture at day 1 in order maximally to inhibit monocyte development because of TLR2 expression (Fig. 3), and sTLR2 partially neutralized HCV proteins inhibition as we documented previously in experiments with human Kupffer cells (16). A more sustained NF-κB induction was observed in the presence of HCV proteins and this could explain the delay in down-regulating the cell surface expression of TLR2 and CD14.

Impaired allostimulatory function of DCs has been reported in individuals with chronic HCV infection (4, 5, 7). Here, we asked whether HCVc and NS3 could be partly responsible for this observation. We demonstrated that HCVc and NS3 inhibit the allostimulatory capacity of DC, suggesting a coinhibitory effect of PD-L1 expression on DCs ability to stimulate T cells (23). These studies are compatible with recent data showing that PBMC-derived DCs from HCV patients with high viral load were functionally impaired, both in terms of phenotype and in terms of allostimulatory capacity (24). Although many studies have reported that IL-10 is involved in tolerogenic DC differentiation (25), our study shows that IL-10 is not the major mechanisms by which HCV proteins inhibited DC development (Fig. 5). However, another study showed that DCs isolated from HCV patients were defective in their capacity to activate CD4+ T cells, and this was alleviated by inhibition of IL-10 (26). We must therefore conclude that HCV targets both differentiation and function in PBMC-derived DCs, albeit through different mechanisms.

The effect of HCV-encoded proteins on DCs is controversial. Thus, a study tested the effects of HCVc, E1 and E2 proteins, delivered to monocyte-derived DCs using an adenoviral vector, and found no effects on several aspects of DC function, including LPS-induced expression of maturation markers and IL-12 (27). The reason for these discrepant results is not entirely clear, but it is possible that the engagement of intracellular TLRs by the vector DNA bypassed the effects of HCVc, which in human Kupffer cells specifically subverts the TLR3 (dsRNA) response (16).

While HCVc and NS3 proteins did not appear to affect the later steps of DC development, HCVc could increase IL-6 secretion by both immature and fully differentiated DCs (Fig. 6A). This may have resulted in the differentiation or expansion of CD4+ Tn, 17 cells (Fig. 6B and C), and our mechanistic experiments demonstrated that IL-6 plays a role in induction of CD4+ T-cell IL-17 by HCVc and NS3-pretreated DCs (Fig. 6D and E). However, it is likely that the same cytokines that drive Th17 cell differentiation can, under slightly different conditions, result in expansion of CD4+ FoxP3+ T-reg cells (25). Tn, 17 are a recently defined proinflammatory CD4+ T-helper cell subset that is characterized by the production of IL-17 and whose differentiation is likely dependent upon encounter with antigen-presenting DCs that produce the appropriate polarizing cytokines. In humans, IL-1β in combination with IL-6 or IL-23 is thought to drive the generation of Th17 cells (28, 29), while IL-1, IL-23 (30), and IL-21 (31) promote the production of IL-17 by memory T cells. With regard to HCV infection, Th17 cells have been implicated in liver injury and fibrosis in HCV infection (32) as well as with HBV infection (33).

These data support the effect of HCV proteins on DC maturation and a subsequent bias toward Th17 cells that may
Fig. 5. IL-10 secretion by differentiating monocytes (A) and (B). Purified monocytes were cultured in RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 IU ml⁻¹ penicillin and streptomycin, supplemented with different concentrations of HCVc or NS3 in the presence or absence of 400 IU ml⁻¹ GM-CSF and 400 IU ml⁻¹ IL-4. After 1 day, 2 days, and 5 days culture, IL-10 production was assayed. Anti-IL-10 mAb was used to block IL-10 secretion by differentiating monocytes (C). Cell phenotypes were analyzed after 1-day culture. These results represent one of three similar experiments.

Table 1. Cytokine levels in DCs differentiation cultures

<table>
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<tr>
<th>Cytokine levels (pg ml⁻¹) (n = 5)</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IFNγ</th>
<th>TNFα</th>
<th>IL-10</th>
<th>IL-12</th>
<th>IL-13</th>
<th>IL-2</th>
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<tr>
<td>iDCs</td>
<td>5.13 ± 1.31</td>
<td>31.96 ± 11.51</td>
<td>16.76 ± 3.45</td>
<td>51.04 ± 8.13</td>
<td>3.59 ± 0.98</td>
<td>2.86 ± 0.19</td>
<td>2.5 ± 0.76</td>
<td>25.42 ± 11.25</td>
</tr>
<tr>
<td>iDC HCVc</td>
<td>13.64 ± 4.12</td>
<td>844.58 ± 54.36</td>
<td>25.28 ± 8.3</td>
<td>64.69 ± 25.34</td>
<td>13.07 ± 5.67</td>
<td>6.73 ± 2.48</td>
<td>5.14 ± 2.3</td>
<td>27.64 ± 15.34</td>
</tr>
<tr>
<td>mDCs</td>
<td>33.96 ± 9.37</td>
<td>27.34 ± 8.69</td>
<td>28.11 ± 13.86</td>
<td>4788.09 ± 1011</td>
<td>4.89 ± 1.52</td>
<td>1.75 ± 0.32</td>
<td>4.29 ± 2.4</td>
<td>29.37 ± 12.3</td>
</tr>
<tr>
<td>mDCs HCVc</td>
<td>27.02 ± 12.3</td>
<td>459.82 ± 63.4</td>
<td>28.19 ± 6.78</td>
<td>2860.71 ± 768.2</td>
<td>9.16 ± 7.35</td>
<td>5.47 ± 1.68</td>
<td>4.15 ± 1.22</td>
<td>29.61 ± 14.63</td>
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*aPeripheral blood monocytes were induced by GM–CSF and IL-4 to differentiate into iDCs in the presence or absence of HCVc for 5 days and then into mDCs by TNF-α for 2 days. Cytokines assay by cytokine bead assay.

*P < 0.05 for cytokine levels of iDCs or mDCs versus HCVc-treated iDCs or mDCs in each tissue analyzed.
be implicated in liver injury related to the viral infection. Taken together with our previous results (16) that demonstrate a subversion of immune responses by KCs toward HCV proteins, we have suggested a model whereby the viral proteins prevent early virus elimination, thus promoting persistence as well as possibly contribute to altering the T-cell response thereby increasing immune-mediated damage. One limitation of these studies is that we have examined the effects of viral proteins in isolation and thus have not accurately replicated the situation of an actual viral infection in the host. We did demonstrate that at least one viral protein, HCV E, had no effect on DC maturation. However, in future studies, the interaction of all viral proteins will obviously have to be examined in a more complex model that replicates the condition of infection.

In summary, these studies add to the understanding of immune subversion and pathology by HCV. Among these effects, HCVc and NS3 both modify peripheral blood monocyte differentiation to interfere with their maturation into DCs. In Kupffer cells, these proteins disable antiviral mechanisms but do not suppress inflammation per se (16). Similarly, HCV-influenced DCs favor the differentiation of Th17 cells, which are active in immune pathology.

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Contribution: Z.T., I.N.C. and M.S.O. designed the research, interpreted data and wrote the paper; Z.T., H.K.H., C.N. and Y.K. performed experiments and Z.T., I.N.C. and M.S.O. analyzed results and made figures.

Conflict of interest disclosure: The authors declare no competing financial interests.

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differentiate into iDCs and mDCs in the presence or absence of different concentrations of HCVc. The supernatants were collected at different time points from cultures of DCs. IFN-γ and IL-17 production from naive CD4 T cells (B and C). IL-6 promotes naive CD4 T-cells IL-17 production in DCs-CD4 T cells co-culture system (D and E). Peripheral blood monocytes were induced by GM-CSF and IL-4 to differentiate into iDCs in the presence or absence of HCV-encoded proteins at concentrations ranging from 0 to 20 μg ml⁻¹ (B) for 5 days and then these DCs were induced to undergo maturation by TNF-α for 2 days. DCs were co-cultured with autologous CD4+ T cells at a 1:1 ratio in the presence or absence of IL-6 or anti-IL-6 antibody (D) for 6 days. The cells were re-stimulated with phorbol myristate acetate/ionomycin followed by brefeldin A for 5 h. Intracellular IFN-γ and IL-17 staining were used to analyze the cells' polarization. The percentage of IL-17+ CD4+ T cells was calculated based on the quadrant gates drawn as shown (C: n = 3; E: n = 5; **P < 0.01, *P < 0.05).

Fig. 6. IL-6 secretion by immature and fully differentiated DCs (A). Purified monocytes were induced by GM-CSF/IL-4 and TNF-α to