Differential Effects of Hepatitis C Virus JFH1 on Human Myeloid and Plasmacytoid Dendritic Cells[▽]

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Dendritic cells (DCs) are reported to be functionally deficient during chronic hepatitis C virus (HCV) infection. Differing results have been reported on direct effects of intact replicative-form HCV on DC function. To better understand the effect of HCV on DC function, we treated freshly purified human myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) with HCV JFH1. We found that HCV upregulated mDC maturation marker (CD83, CD86, and CD40) expression and did not inhibit Toll-like receptor 3 (TLR3) ligand [poly(I:C)]-induced mDC maturation, a finding consistent with the phenotype of DCs from HCV-infected subjects. At the same time, HCV JFH1 inhibited the ability of poly(I:C)-treated mDCs to activate naive CD4 T cells. In contrast, although there was no direct effect of virus on pDC maturation, HCV JFH1 inhibited TLR7 ligand (R848)-induced pDC CD40 expression, and this was associated with impaired ability to activate naive CD4 T cells. Parallel experiments with recombinant HCV proteins indicated HCV core protein may be responsible for a portion of the activity. Furthermore, HCV-mediated mDC maturation was dependent upon CD81-E2 interaction and, in part, TLR2. Using UV-treated HCV, we show that HCV-mediated mDC and pDC maturation is virus replication independent and, using strand specific PCR, we found no evidence for HCV replication within DCs. Because these effects of HCV on DC subset maturation and function in part recapitulate direct ex vivo analysis of DCs in chronic HCV infection, the mechanisms described here likely account for a portion of the DC subset defects observed in vivo.

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family of viruses and is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (19, 32). Globally, it is estimated that roughly 180 million people (3% of the population) are infected with HCV (13). Although cellular and humoral immune responses are present during acute and chronic HCV infection, the immune response is low in efficiency for elimination of infection (12, 33, 37). While CD4⁺ and CD8⁺ T-cell responses play a critical role in virus clearance during acute infection, the means by which HCV escapes host immune surveillance during chronic infection is less clear. In addition, during chronic infection host immune impairment exists, as response to neoantigen challenge in the form of vaccine is impaired (22, 28, 52). One possible explanation for immune impairment during chronic infection is a defect in antigen-presenting cell (APC) function.

Dendritic cells (DCs) are APCs that are highly potent in naive T-cell activation. In the peripheral blood, two major DC populations can be identified: CD11C⁺ myeloid DCs (mDCs) and CD123⁺ plasmacytoid DCs (pDCs) (5, 36). mDCs are derived from a myeloid bone marrow precursor, produce large amounts of interleukin-12 (IL-12) after bacterial or viral infection and prime Th1 T-cell responses (5). pDCs are thought to play a central role during the antiviral response through producing large amounts of alpha interferon (IFN- α) (21, 36).

mDCs and pDCs have different patterns of TLR expression. mDCs express Toll-like receptor 1 (TLR1) to TLR6, TLR8, TLR10, and possibly TLR7, while pDCs express TLR1, TLR6, TLR10, and very high levels of TLR7 and TLR9 (25–27, 31).

Several studies have shown the stimulatory capacity of monocyte-derived DCs (MoDCs) or mDC to be impaired in chronic HCV infection (3, 4, 29). Possible mechanisms for this finding include reduced DC frequencies, increased IL-10 production, reduced IL-12 production, and loss of maturation capacity—all of which may be secondary to direct DC infection or indirectly mediated by soluble factors (2–4, 29, 49, 51). HCV structural and nonstructural proteins have also been reported to inhibit DC maturation and function (16, 46). In contrast, other studies have identified no defect in MoDC IL-12 production or T-cell stimulatory function (38, 39, 42). Similarly, differing results have been reported on pDC function in chronic HCV infection, with some studies finding decreased pDC frequency, IFN- α production, and T-cell stimulatory capacity, while others have found no defects (2, 38, 39, 42, 49, 51, 54).

Recently, an in vitro cell culture system for HCV has been successfully developed (34, 50, 53, 56). Differing results have been reported regarding the effect of cell-cultured HCV (HCVcc) on DC function. Some reports describe an inhibitory effect of HCVcc on MoDC maturation and activation of antigen-specific T cells or TLR9 ligand-mediated pDC IFN- α production (45, 48), while other studies have found no defects in HCVcc-treated mDCs, MoDCs, or pDCs (14, 18, 48). In some studies, DC defects were thought to be in part attributable to JFH1-infected cell apoptosis (17, 48). These discrepancies may

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result from differences in HCVcc (dose, genotype, or culture condition), DC source (mDCs or MoDCs), or functional read-out. To further explore the direct effect of HCV on DC subset function and to identify mechanisms involved, we exposed freshly isolated, nonexpanded mDCs and pDCs to HCV (JFH1) or recombinant HCV proteins and evaluated the DC maturation, TLR ligand-induced DC maturation and cytokine production, and the capacity of DCs to activate naive CD4 T cells. Our results indicate that HCV inhibited TLR7 ligand-mediated pDC CD40 expression, while upregulating mDC CD83, CD86, and CD40 expression in a virus replication-independent manner. The latter effects are partially mediated by CD81/HCV E2, TLR2, and HCV core protein. Furthermore, the presence of HCV impaired TLR ligand-mediated mDC and pDC activation of naive CD4 T cells, and this functional impairment may be partially mediated by HCV core protein. These data extend our understanding of the mechanisms and effects of HCV directly on DC subsets and provide information that in part explain differences in prior study findings (14, 18, 45, 48).

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line Huh7.5 was provided by C. M. Rice (Apath LLC, St. Louis, MO) and was maintained in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% nonessential amino acid, 100 U of penicillin/ml, and 100 µg of streptomycin/ml.

Production and titration of infectious HCV JFH1. HCV JFH1, a genotype 2a HCV strain, was grown on Huh7.5 cells. The pJFH1 plasmid was provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan). pJFH1 plasmid was digested with XbaI, and the linearized DNA was used for *in vitro* transcription of HCV plus-strand RNA with a MEGAscript T7 kit (Ambion, Austin, TX). Huh7.5 cells were washed twice with the reduced serum medium Opti-MEM 1 (Invitrogen) and then transfected with 10 µg of JFH1 RNA using DMRIC-C (Invitrogen). Infectious JFH1 virus production was measured by transferring supernatant from transfected cells to naive Huh7.5 cells, followed by culture and then staining for HCV core expression by immunofluorescence using monoclonal mouse anti-HCV core (5 µg/ml; Anogen, Mississauga, Ontario, Canada) and goat anti-mouse immunoglobulin G conjugated to Alexa Fluor 488 (20 µg/ml; Invitrogen, Eugene, OR). Infected cells were cultured for 3 passages to achieve maximal infectivity (44). The concentration of stock virus was 2×10^6 focus-forming units/ml as determined by titration, 4×10^6 fmol of HCV core/liter as determined by an Ortho HCV core antigen enzyme-linked immunosorbent assay (ELISA; Ortho Clinical Diagnostics, Tokyo, Japan), and 2×10^8 RNA copies/ml as determined by a COBAS AmpliScreen HCV test (v2.0; Roche, Pleasanton, CA). Infectious supernatants were filtered through 0.45-µm-pore-size filter units, divided into aliquots, and stored at -80°C for DC experiments. Supernatants from uninfected Huh7.5 cells were used as a mock control. In some experiments, UV-induced apoptotic Huh7.5 cell supernatants were also used as a control.

Cell isolation. mDCs and pDCs were isolated from fresh peripheral blood mononuclear cells (PBMC) of healthy control donors using CD19 depletion, followed by CD1c DC-positive selection (for mDCs) or BDCA-4-positive selection (for pDCs) (Miltenyi Biotec, Auburn, CA) using sequential separation on AutoMacs (Miltenyi Biotec). Naive CD4 T cells were isolated by negative selection removing CD8-, CD14-, CD16-, CD19-, CD36-, CD45RO-, CD56-, CD123-, TCRγδ-, and glycoporin A-expressing cells (Miltenyi Biotec). Cells were cultured in complete RPMI with 5% human AB serum (Gemini Bio-Products, Woodland, CA), 2 mmol of L-glutamine/liter, 100 U of penicillin/ml, and 100 µg of streptomycin/ml. All studies were approved by the institutional review boards at the Cleveland Veterans Affairs Medical Center and the University Hospitals of Cleveland.

HCV JFH1-treated mDC and pDC maturation. Freshly isolated mDCs or pDCs (3×10^4 cells/well) were cultured in 96-well plates for 20 h with HCV JFH1 at a multiplicity of infection (MOI) of 10 as determined on Huh7.5 cells, with HCV JFH1 at an MOI of 1, with the supernatant from uninfected (mock-infected control) Huh7.5 cells, or with the UV-induced supernatant from Huh7.5 cells. In some experiments, JFH1 was treated with UV (254 nm using a 60-Hz, 0.16-A UV lamp at a distance of 6 cm for 1 min) to inactivate the infectivity (the

time curve of UV treatment indicated that a 1-min treatment resulted in sub-maximal [90%] inactivation of infectivity for Huh7.5 cells). To detect the effect of HCV JFH1 on TLR ligand-induced DC maturation, mDCs were cultured with HCV JFH1 in the presence or absence of poly(I:C) (50 µg/ml; Amersham Bioscience), while pDCs were cultured in the presence or absence of R848 (1 µg/ml; InvivoGen) or CpG 2216 (10 µM; Integrated DNA Technologies). To determine the effect of recombinant HCV proteins on DC maturation, recombinant HCV core (amino acids [aa] 2 to 120), NS3 (aa 1192 to 1457), NS4 (aa 1569 to 1931), or NS5 (aa 2054 to 2995) protein (10 µg/ml; Chiron, Emeryville, CA) was added to mDCs or pDCs in the presence or absence of TLR ligand [poly(I:C) for mDCs and R848 for pDCs]. After 20-h culture, supernatants were collected for cytokine detection, while cells were harvested for cell surface CD83, CD86, and CD40 expression as determined by flow cytometry. Cells were washed with phosphate-buffered saline containing 0.01% bovine serum albumin and stained for surface marker expression of CD11c (BD Biosciences) for mDCs and BDCA-2 (Miltenyi Biotec) for pDCs. For DC maturation expression of CD83-fluorescein isothiocyanate (FITC) (clone HB15e), CD86-phycoerythrin (clone IT2.2) and CD40 phycoerythrin-Cy5 (clone 5C3) were detected. DC expression of CD81 and SR-BI was measured by using CD81-FITC (clone JS-81; BD Biosciences) and polyclonal anti-SR-BI with secondary goat anti-rabbit immunoglobulin G-FITC (Novus Biologicals and eBioscience, respectively). Stained cells were analyzed on a FACScalibur (BD Biosciences) flow cytometer with CellQuest software (BD Biosciences).

For analyzing the effect of blocking antibody on DC maturation, mDCs were incubated with monoclonal anti-CD81 (clone JS-81, 10 µg/ml; BD Biosciences), anti-E2 (20 µg/ml; BiotDesign International), anti-TLR2 (clone T2.5, 10 µg/ml; eBioscience), polyclonal anti-SR-BI (1:1,000; Novus Biologicals), or isotype control for 1 h. pDCs were treated with anti-CD81 or isotype control for 1 h. JFH1 was added, and the culture was continued for 20 h at 37°C . Cells were analyzed for maturation marker CD83, CD86, and CD40 expression.

Analysis of mDC and pDC maturation state in HCV-infected patients. Chronically HCV-infected subjects ($n = 18$) had detectable serum HCV antibodies and RNA ($n = 15$ [genotype 1], $n = 1$ [genotype 3], and $n = 2$ [genotype unknown]). No subjects had previously received therapy for HCV. Flow cytometry was performed on a LSRII (BD Biosciences, San Jose, CA) flow cytometer with CellQuest Software (BD Biosciences) using freshly prepared PBMC (2×10^6 PBMC analyzed per sample). All stains were performed in the dark for 20 min at 25°C , and then cells were washed in phosphate-buffered saline with 0.01% bovine serum albumin, fixed in 1% paraformaldehyde, and stored at 4°C until analysis. PBMC were stained with either (i) anti-BDCA-1 or anti-CD19 monoclonal antibodies (MAbs; Miltenyi Biotec and BD Biosciences) for mDC analysis or (ii) anti-BDCA-2 MAb (Miltenyi Biotec) for pDC analysis. All PBMC were stained with anti-HLA-DR, anti-CD83, anti-CD86, and anti-CD40 MAbs. Live cells were identified by forward- and side-scatter analyses. mDCs were gated as CD19 $^-$ BDCA-1 $^+$ HLA-DR $^+$ cells, while pDCs were gated as BDCA-2 $^+$ HLA-DR $^+$ cells. For each DC subset CD83, CD86, and CD40 expression was determined by mean fluorescence intensity (MFI).

IFN-γ ELISPOT assay of naive T-cell activation. Freshly isolated mDCs or pDCs (10^4 cells/well) were preincubated with JFH1 at an MOI of 10 or with recombinant HCV core, NS3, NS4, or NS5 protein at 10 µg/ml in the presence or absence of poly(I:C) (for mDCs) or R848 (for pDCs) for 4 h, and then allogeneic naive CD4 T cells (2×10^5 cells/well) were added to each well and cultured for a total of 72 h at 37°C . At 48 h, cells were transferred to enzyme-linked immunosorbent assay (ELISPOT) plates (Whatman) precoated with IFN-γ capture antibody (4 µg/ml; Endogen). Naive CD4 T cells were obtained from two constant allogeneic donors (cells from one donor were used for evaluating all mDC-dependent naive CD4 T-cell activation, and cells from the other donor were used for evaluating all pDC-dependent naive CD4 T-cell activation). In some experiments, DCs were washed twice after a 4-h preincubation with virus to get rid of any virus or protein not cell associated and then treated as described above. Culture supernatants were analyzed for IL-2 and IL-10 by ELISA.

For analyzing the effect of blocking antibody on DC-mediated naive CD4 T-cell activation, DCs were pretreated with anti-IL-10 (10 µg/ml; R&D Systems), anti-CD81, anti-TLR2 (mDCs only), or anti-SR-BI for 1 h, followed by IFN-γ ELISPOT assay as discussed above.

Analysis of cytokine production. Cell supernatants were harvested for cytokine analysis by ELISA according to the manufacturer's standard protocols (IL-2, IL-6, IL-10, IL-12, and TNF-α from eBioscience; IFN-α from PBL Biomedical Laboratories, Piscataway, NJ).

Analysis for infection and replication in HCV JFH1-treated mDCs and pDCs. Purified mDCs or pDCs were cultured with HCV JFH1 at an MOI of 10 for 24 or 72 h. Cells were washed three times to get rid of any dissociative virus. Virus RNA was isolated from cells by using RNeasy minikit (Qiagen, Hilden, Ger-

A mDC **B mDC + poly IC** **C pDC** **D pDC+R848**

FIG. 1. Differential effects of HCV JFH1 on mDC and pDC maturation marker CD83, CD86, and CD40 expression in the absence or presence of TLR ligand. Freshly purified mDCs (3×10^4 /well) from different healthy control donors ($n = 15$) were treated with HCV JFH1 at an MOI of 10 or 1 ($n = 9$ donor samples for an MOI of 1) (A) or with poly(I:C) plus HCV JFH1 at an MOI of 10 or 1 ($n = 7$) (B) for 20 h. Similarly, purified pDCs (3×10^4 /well) from different healthy control donors were treated with HCV JFH1 at an MOI of 10 or 1 ($n = 6$) (C) or R848 plus HCV JFH1 at an MOI of 10 or 1 ($n = 10$) (D) for 20 h. Expression of CD83, CD86, and CD40 was evaluated by flow cytometry. The data are shown as MFI median and interquartile range values with a representative result shown in histogram format above. Statistical analysis of expression of indicated maturation marker was compared between HCV JFH1-treated DCs and mock-treated DCs (A and B) or between HCV JFH1 plus TLR ligand-treated DCs and TLR ligand alone-treated DCs (C and D). Symbols: ○, outlier values (between 1.5 and 3 times the interquartile range); *, extreme values (>3 times the interquartile range).

TABLE 1. Expression of maturation markers on mDCs and pDCs of chronically HCV-infected patients

DC type ^a	CD type	Median MFI (range)		P value
		Control	HCV	
mDC	CD83	530 (352–953)	1,039 (637–2,393)	<0.001
	CD86	2,543 (1,417–4,689)	5,312 (2,812–31,215)	<0.001
	CD40	428 (277–1,668)	1,021 (192–3,167)	0.007
pDC	CD83	255 (174–1,030)	605 (207–1,899)	0.009
	CD86	485 (271–1,673)	1,876 (253–14,856)	0.014
	CD40	282 (55–810)	583 (130–1,798)	0.092

^a For mDCs, *n* = 10 control subjects; *n* = 16 HCV-infected subjects; for pDCs, *n* = 11 control subjects; *n* = 18 HCV-infected subjects.

many). HCV plus and minus-strand RNA was detected by strand-specific reverse transcription-PCR as described Blackard et al. (8).

Statistical analysis. We used conventional measures of central tendency and dispersion to describe the data. We compared categorical variables by using the Fisher exact test, independent continuous variables by using the Mann-Whitney U test, and values in the presence or absence of various stimuli by using the Wilcoxon signed-rank test. To assess the association among continuous variables, we used the Spearman's rank correlation coefficient. All comparisons are double-sided, and a *P* value of 0.05 was considered nominally significant.

RESULTS

HCV JFH1 enhances mDC expression of CD83, CD86, and CD40, while there is no direct effect of HCV on pDC maturation. To explore the direct effect of HCV on DC maturation state, mDCs and pDCs were cultured with JFH1 at an MOI of 10 and an MOI of 1 for 20 h. Surface markers of DC maturation were analyzed by flow cytometry. As shown in Fig. 1A, significant upregulation of CD83, CD86, and CD40 was observed on mDCs exposed to JFH1. The effect is concentration dependent, because increased expression of CD83, CD86, and CD40 was observed for HCV JFH1 (*P* = 0.002, 0.003, and 0.005, respectively) at an MOI of 10, but not at for HCV JFH1 at an MOI of 1. In contrast, the presence of HCV JFH1 had no effect on pDC maturation at an MOI of either 10 or 1 (Fig. 1C). These results indicate replicative-form HCV has differential effects on mDC and pDC maturation. Because HCV JFH1-induced HUH apoptosis has been previously reported (17, 48), it is possible that HCV JFH1-associated HUH apoptotic factors participate in the HCV JFH1-mediated effect. Additional experiments with UV-induced apoptotic Huh7.5 cell supernatants revealed no direct effect on DC maturation, indicating soluble factors related to Huh7.5 apoptosis do not likely play a role here (results not shown).

The effect of HCV JFH1 on mDC and pDC maturation appeared to recapitulate what we have previously observed in samples freshly prepared from chronic HCV-infected individuals (54). Because previous assays for CD83 and CD86 expression were performed on isolated DCs, potentially introducing activation stimulus at time of DC isolation, we analyzed another group of chronic HCV-infected individuals for mDC and pDC maturation state using unfractionated PBMC and flow cytometry. We again found results similar to those of our prior study (54). Specifically, mDCs from HCV-infected patients show increased expression of CD83, CD86, and CD40 (Table 1). These results indicate that the effect we observe with in vitro HCV JFH1 treatment of mDCs is consistent with what

occurs in vivo. In contrast, pDCs from HCV-infected patients show increased expression of CD83 and CD86, differing from the in vitro culture results with HCV JFH1, perhaps indicating that indirect effects of virus on pDCs are operative in vivo.

TLR ligand-induced mDC and pDC maturation and cytokine production are differentially affected by HCV JFH1. Since DCs are commonly activated via TLR ligands, we evaluated the effect of replicative-form HCV on TLR ligand-induced DC maturation. We used the TLR3 ligand poly(I:C) to stimulate mDCs and the TLR7 ligand R848 to stimulate pDCs. These ligands were chosen based upon optimal activity in assays of TLR ligand-dependent naive CD4 T-cell activation (54). As shown in Fig. 1B, poly(I:C) stimulation of mDCs resulted in significantly increased CD83, CD86, and CD40 expression, and the presence of HCV JFH1 did not inhibit this maturation process. In fact, CD86 expression was further increased by the presence of HCV JFH1 at an MOI of 10. On the other hand, HCV JFH1 inhibited R848-induced pDC CD40 expression (Fig. 1D). The inhibitory effect was observed at MOIs of both 10 (*P* = 0.047) and 1 (*P* = 0.025).

To detect whether the presence of HCV affects DC cytokine production in response to TLR ligand, we measured culture supernatants of HCV JFH1 and TLR ligand-treated DCs for cytokine production (for mDCs, IL-12, IL-10, IL-6, and TNF- α and for pDCs, IFN- α , IL-10, IL-6, and TNF- α). HCV JFH1 had no effect on mDC TNF- α or IL-6 production in the presence or absence of poly(I:C) (Fig. 2A). No mDC IL-12 or IL-10 production was detected in these cultures (data not shown). R848-induced pDC IFN- α was also not affected by the presence of HCV JFH1. At the same time the presence of HCV JFH1 enhanced R848-induced pDC TNF- α production (Fig. 2B). These results suggested that HCV JFH1 enhances TLR ligand-induced pDC secretion of select cytokines while having no observed effect on TLR ligand-induced mDC cytokine secretion.

TLR ligand-dependent mDC and pDC activation of naive CD4 T cells is inhibited by the presence of HCV JFH1. Since dendritic cells are unique in their potent ability to activate naive T cells and since chronic HCV infection is associated with impairment in ability of DCs to activate T cells (2, 4, 29, 49, 54), we next analyzed the direct effect of HCV JFH1 on TLR ligand-dependent DC activation of naive CD4 T cells. In the absence of TLR ligand, mDC-dependent naive CD4 T-cell activation was observed, and the presence of HCV JFH1 inhibited mDC-dependent T-cell IL-2 (*P* = 0.011, Fig. 3D) and tended to inhibit mDC-dependent T-cell IFN- γ (*P* = 0.126, Fig. 3A and B). Similarly, the presence of HCV JFH1 inhibited pDC-dependent naive CD4 T-cell IFN- γ (*P* = 0.017, Fig. 3A and C). Very little pDC-dependent naive CD4 T-cell IL-2 was observed (Fig. 3E).

The presence of TLR ligand enhanced the ability of both mDCs and pDCs to activate naive CD4 T cells, and this activation was also inhibited by the presence of HCV JFH1 (mDCs, *P* = 0.037; pDCs, *P* = 0.008) (Fig. 3A, B, and D). No IL-10 production was detected in either mDC or pDC stimulated T-cell supernatants. To address the possible direct inhibitory effect of HCV JFH1 on T-cell activation, we washed mDCs and pDCs twice after a 4-h preincubation with HCV JFH1 (before the addition of naive CD4 T cells) in order to remove virus not associated directly with DCs. After the wash-

FIG. 2. Differential effects of HCV JFH1 on mDC and pDC cytokine expression. mDCs and pDCs were cultured as described in Fig. 1. Secreted cytokines were quantified from culture supernatants by ELISA. (A) Cytokine secretion (TNF- α and IL-6) by mDCs treated with or without HCV JFH1 and poly(I:C). (B) Cytokine secretion (TNF- α , IL-6, and IFN- α) by pDCs treated with or without HCV JFH1 and R848. The data are shown as cytokine concentration median and interquartile range values. Statistical analyses of indicated cytokines were compared between HCV JFH1-treated DCs and mock-treated DCs or HCV JFH1 plus TLR ligand-treated DCs and TLR ligand alone-treated DCs.

TABLE 2. HCV core protein effect on DC maturation^a

DC type	CD type	Median MFI (range)		<i>P</i> value
		Mock	HCV core	
mDC	CD83	32 (26–60)	38 (32–58)	0.011
No poly(I:C)	CD86	81 (19–122)	124 (29–200)	0.008
	CD40	35 (26–53)	60 (34–142)	0.008
Poly(I:C)	CD83	36 (18–299)	32 (20–196)	>0.05
	CD86	176 (70–850)	223 (59–1,094)	0.005
	CD40	62 (2–127)	49 (4–174)	>0.05
pDC	CD83	819 (17–1,104)	881 (22–1,165)	>0.05
No R848	CD86	658 (19–1,406)	686 (23–2,083)	>0.05
	CD40	404 (31–770)	420 (37–650)	>0.05
R848	CD83	28 (13–201)	44 (23–313)	>0.05
	CD86	86 (45–324)	53 (22–445)	>0.05
	CD40	68 (7–109)	28 (5–79)	0.022

^a No significant effects of other HCV proteins (NS3, NS4, and NS5) on mDC and pDC maturation were observed (data not shown).

ing step, HCV JFH1-pretreated DCs still showed decreased naive CD4 T-cell activation activity, indicating that the effect of HCV JFH1 on DC activation of naive T cells requires an interaction between HCV JFH1 and DCs (Fig. 3F and G).

HCV JFH1 activity may be partially mediated by HCV core protein. It has been previously reported that HCV core and NS3 proteins can directly inhibit MoDC maturation and the allostimulatory capacity of immature DCs (16, 46). To determine whether recombinant HCV proteins have inhibitory effects in our system and to identify specific components of the HCV virus particle that may be responsible for the activity of HCV JFH1, we treated mDCs and pDCs with recombinant HCV core, NS3, NS4, and NS5 proteins and evaluated DC maturation and the ability to activate naive CD4 T cells. Treatment of DCs with HCV core protein—but not NS3, NS4, or NS5 proteins—resulted in modest effects similar to treatment with HCV JFH1. As shown in Table 2, HCV JFH1 HCV core protein modestly increased mDC CD83, CD86, and CD40 expression ($P = 0.011$, 0.008 , and 0.008 , respectively) and inhibited R848-induced pDC CD40 expression ($P = 0.022$). HCV core protein, but not HCV NS3, NS4, or NS5 protein, also modestly impaired TLR ligand-dependent mDC and pDC activation of naive CD4 T cells (mDCs, $P = 0.008$; pDCs, $P = 0.041$) (Fig. 4). However, in contrast to HCV JFH1, HCV core protein inhibited R848-induced pDC IFN- α production ($P = 0.008$) and poly(I:C)-induced mDC IL-6 production ($P = 0.009$) (Table 3). These effects were concentration dependent (data not shown). Together, these findings indicate HCV core protein, but not the other HCV proteins analyzed, has activity

similar to that of HCV JFH1 on DC subsets, suggesting that HCV core protein may be in part responsible for the HCV JFH1 mediated activity. Notably, HCV E2 protein was not investigated in these experiments.

HCV JFH1-induced mDC maturation may be partially mediated by TLR2. HCV core protein has been shown to interact with TLR2 and facilitate MoDC defects (49). mDCs are also known to express TLR2 (24). We therefore specifically evaluated whether TLR2 was involved in the direct effects of HCV JFH1 on mDCs. In control experiments, anti-TLR2 blocking antibody inhibited TLR2 ligand (pam3cys)-induced mDC maturation (Fig. 5A). This antibody also partially inhibited HCV JFH1-induced mDC CD40 expression ($P = 0.027$; Fig. 5B). In addition, anti-TLR2 antibody partially inhibited HCV JFH1-induced CD86 expression ($P = 0.028$), whereas there was no significant inhibition of HCV JFH1-induced CD83 expression (data not shown), suggesting a modest but partial role for TLR2 in HCV JFH1-mediated mDC maturation. At the same time, TLR2 blockade did not abrogate the inhibitory effect of HCV JFH1 on mDC-mediated naive CD4 T-cell activation (data not shown), suggesting a different mechanism of DC activation and inhibition of DC-mediated T-cell activation.

HCV JFH1-induced mDC maturation is dependent on CD81. CD81 and scavenger receptor B type 1 (SR-B1) are required for HCV entry into hepatoma cell lines (11, 30, 43, 47, 55). It has also been previously suggested that SR-B1 is involved in the MoDC uptake of replicative HCV (7). To identify whether either of these receptors are required for HCV effects in our system, we first evaluated whether freshly isolated mDCs or pDCs express either of these receptors. As shown in Fig. 6A, mDCs express CD81 and SR-B1, indicating these may be viable receptors for HCV JFH1-mediated DC activity. We next evaluated whether antibody blockade of CD81 or SR-B1 abrogated HCV JFH1 effects upon DC subsets. As expected, control assays indicated that antibody blockade of either CD81 or SR-B1 inhibits HCV JFH1 infection of Huh7.5 cells (Fig. 6B). Additional control assays showed no toxic or inhibitory activity of these antibodies upon TLR ligand-induced DC activity (results not shown). Anti-CD81, but not anti-SR-B1, blocked HCV JFH1-induced CD40 expression ($P = 0.042$; Fig. 6C). In addition, anti-CD81 blocked HCV JFH1-induced mDC CD83 and CD86 expression ($P = 0.041$ and 0.043 , respectively; data not shown). In contrast, HCV JFH1-mediated inhibition of TLR ligand-dependent mDC activation of naive CD4 T cells was not abrogated by CD81 blockade (Fig. 6D), again suggesting differing mechanisms for effects of HCV JFH1 on mDC maturation versus mDC-mediated T-cell activation. Because HCV interacts with CD81 via E2-CD81 inter-

FIG. 3. Effects of HCV JFH1 on TLR ligand-independent and -dependent mDC and pDC activation of naive CD4 T cells. mDCs and pDCs from healthy control donors (10^4 /well, $n = 10$) were preincubated with or without HCV JFH1 at an MOI of 10 in the presence or absence of TLR ligand for 4 h and cultured with purified naive CD4 T cells (2×10^5 /well) for 72 h. One representative IFN- γ ELISPOT assay is shown in panel A. Summative data are shown for mDC (B) and pDC (C) activation of naive CD4 T cells to expand and produce IFN- γ by ELISPOT assay. The data are shown as spot-forming units (SFU)/well. Symbols: $\circ$, outlier values (between 1.5 and 3 times the interquartile range); *, extreme values (values that are >3 times the interquartile range). mDC (D)- and pDC (E)-activated naive CD4 T-cell IL-2 was detected from cultured supernatants by ELISA. Statistical analysis of HCV JFH1-treated DCs versus mock-treated DCs and of HCV JFH1 plus TLR ligand-treated DCs versus TLR ligand-treated DCs. mDCs (F; $n = 5$) and pDCs (G; $n = 1$, with another experiment revealing similar results) were preincubated with or without HCV JFH1 plus TLR ligand for 4 h, washed twice, and cultured with purified naive allogeneic CD4 T cells for 72 h. CD4 IFN- γ was detected by ELISPOT assay.

FIG. 4. HCV core protein effect on TLR ligand-dependent DC activation of naive CD4 T cells. mDCs (A) and pDCs (B) ($n = 10$) were preincubated with 10 μ g of HCV core, NS3, NS4, or NS5 protein/ml in the presence of TLR ligand for 4 h [poly(I:C) for mDCs; R848 for pDCs] and then cultured with purified naive CD4 T cells for a total of 72 h. IFN- γ production was detected by ELISPOT assay. Statistical analysis of proteins plus TLR ligand-treated DCs versus TLR ligand-treated DCs is represented for P values of ≤ 0.05 .

actions, we also evaluated the dependence of HCV JFH1-induced MDC activation on E2 using blocking antibody. As shown in Fig. 6F, HCV JFH1-induced mDC CD83, CD86, and CD40 expression was partially blocked by anti-E2, suggesting that binding of CD81/E2 is likely required for HCV JFH1-

mediated mDC maturation. The E2 blocking antibody was notably formed against HCV genotype 1 protein, indicating cross-reactivity with genotype 2 HCV here.

Increased IL-10 production from HCVcc-treated MoDCs has been reported to be associated with impaired MoDC abil-

TABLE 3. HCV core protein effect on DC cytokine secretion

DC type	Cytokine	Median concn in pg/ml (range)			<i>P</i> value ^a
		Mock	TLR ligand	TLR ligand + core	
mDC	IL-6	64 (0–697)	303 (52–2,216)	222 (0–1,011)	0.009
	TNF- α	0 (0–120)	0 (0–285)	0 (0–0)	>0.05
pDC	IFN- α	0 (0–223)	631 (0–1,320)	1 (0–248)	0.008
	IL-6	127 (0–1,752)	864 (194–3,876)	760 (117–4,146)	>0.05
	TNF- α	0 (0–297)	76 (0–2,000)	0 (0–1,335)	>0.05

^a That is, the *P* value comparing TLR ligand-treated DCs and DCs treated with TLR ligand plus core protein.

ity to stimulate antigen-specific CD4⁺ and CD8⁺ T cells (45). Although we did not detect any IL-10 production in the supernatants of cultured mDCs, pDCs, or mDC- and pDC-activated CD4 T cells, we considered whether undetectable amounts of IL-10 may have contributed to the impaired naive CD4 T-cell activation. IL-10 blocking antibody, functional in the abrogation of IL-10-mediated inhibition of phytohemagglutinin-stimulated PBMC IL-2 and TNF- α production (data not shown), did not abrogate HCV JFH1-mediated inhibition of TLR ligand-dependent mDC (Fig. 6E) and pDC (data not shown) activation of naive CD4 T cells. In addition, real-time PCR analysis of three healthy control mDC samples in the absence or presence of HCV JFH1 revealed no more than a 1.5-fold increase in IL-10 mRNA level when HCV JFH1-treated mDCs were compared to control treated mDCs (data not shown). We are therefore unable to identify a role for IL-10 in the present system.

Although pDCs also express CD81 at lower levels than do mDCs (data not shown), the effect of HCV JFH1 on TLR7 ligand-induced pDC CD40 expression and pDC activation of naive CD4 T cells was not abrogated by anti-CD81 blocking antibody (results not shown).

Viral replication is not required for the effect of HCV JFH1 on mDC and pDC maturation. It is controversial whether HCV can infect and replicate within human DCs since no evidence of HCV protein expression has been found within DCs (4, 20, 41). Using a stock of UV-treated HCV JFH1 (characterized to have 90% reduction in infectious activity), we found that UV-treated HCV JFH1 has similar effects compared to non-UV treated HCV JFH1 on mDCs (Fig. 7A) and pDCs (Fig. 7B). This is consistent with an activity that is viral replication independent.

As an additional means of evaluating whether infection of DCs is required for the effects observed, PCR-based analysis for plus- and minus-strand HCV revealed no detectable minus-strand HCV RNA in seven different mDC samples treated with HCV JFH1, whereas minus-strand HCV RNA was readily detectable in HCV JFH1-treated Huh7.5 cells (not shown). At the same time plus strand HCV RNA was detected in five of seven mDC samples treated with HCV JFH1 at an MOI of 10 for 20 and 72 h. Plus- and minus-strand HCV RNA was identified in one of four pDC samples, indicating that HCV may infect a portion of pDCs (not shown). Taken together, these data indicate that while HCV may enter mDCs, we find no evidence for virus replication, and virus replication is not necessary for HCV JFH1-mediated effects on mDCs or pDCs.

DISCUSSION

Dendritic cells are responsible for initiation and regulation of immune responses (6, 35). We and others have described mDC and pDC numerical and functional defects during chronic HCV infection (2–4, 29, 49, 54). In particular, we have previously identified a state of enhanced mDC maturation in the setting of chronic HCV infection (54) and an impairment in TLR ligand-dependent pDC activation of naive CD4 T cells (54). The results presented here indicate that replicative-form HCV can directly activate mDCs to express markers also observed to be enhanced in expression during chronic HCV infection. This effect is likely in part mediated by HCV core protein, is dependent on CD81 and HCV E2, and appears to be partially dependent on TLR2. At the same time, HCV has an inhibitory effect on mDC-mediated naive T-cell activation. The latter activity does not appear to be CD81 or TLR2 dependent and was not observed in our previous study of mDCs freshly prepared from HCV-infected individuals (54), suggesting a different mechanism of effect. pDC assays indicated no direct HCV JFH1-mediated activation of pDCs. At the same time, TLR7 ligand-mediated pDC CD40 expression and activation of naive CD4 T cells are inhibited by the presence of HCV JFH1, an observation consistent with our previous findings of cells derived from chronically HCV-infected individuals (54). Overall, we show here that HCV directly activates mDCs in a CD81- and TLR2-dependent manner and yet inhibits TLR ligand-dependent mDC activation of naive CD4 T cells, whereas TLR ligand-induced pDC activation and pDC-mediated naive CD4 T-cell activation are inhibited by HCV. Since HCV core protein alone tends to provide similar activities, a portion of HCV JFH1-mediated activities may be attributed to HCV core protein.

With the recently developed system for replicative-form, in vitro cell-cultured HCV, four recent reports present somewhat differing results about the effect of HCV on mDC or MoDC and pDC function, with some studies suggesting HCV-mediated inhibition versus no effect on DC maturation or the capacity to activate T cells (14, 18, 45, 48). Our results of HCV JFH1-induced mDC maturation (upregulation of CD83, CD86, and CD40 expression) and yet no effect on TLR ligand-induced mDC maturation or cytokine secretion are entirely consistent with our current (Table 1) and previous (54) ex vivo data of mDCs from HCV-infected patients. This suggests HCV may affect mDCs by mechanisms observed here in our in vitro experiments. Saito et al. (45) recently reported that HCV

FIG. 5. Anti-TLR2 partially blocks HCV JFH1-induced mDC maturation. (A) Effect of anti-TLR2 antibody on pam3cys-induced mDC maturation. mDCs were incubated with 10 μ g of anti-TLR2 MAb/ml or isotype control for 1 h, 20 ng of pam3cys/ml was added, and the culture was continued for 20 h. Maturation marker expression was detected by flow cytometry. (B) Anti-TLR2 partially blocks HCV JFH1-induced mDC maturation. Purified mDCs were preincubated with anti-TLR2 for 1 h and cultured with HCV JFH1 at an MOI of 10 for 20 h. Maturation marker expression was evaluated by flow cytometry. The data are shown as the mean and standard deviation of five healthy controls. Paired nonparametric statistical analysis is shown.

JFH1 inhibits maturation cocktail (containing IL-1 β , IL-6, TNF- α , and prostaglandin E₂)-induced MoDC maturation. Such differences between that study and the results presented here may be attributable to differing maturation stimuli [poly(I:C) versus maturation cocktail] or different cell populations (mDCs versus MoDCs). Certainly, MoDCs and mDCs have been found to differ in a number of characteristics, in-

cluding maturation status, cytokine secretion, and the ability to activate T cells (23). Shiina and Rehermann (48) and Ebihara et al. (18) have recently reported no observed effect of HCV JFH1 on mDC or MoDC maturation or their ability to activate T cells. Differences between the latter studies and the present one may be attributable to a number of factors, including different cell populations studied (purified mDCs versus

FIG. 6. Anti-CD81 blocks HCV JFH1-induced mDC maturation. (A) CD81 and SR-BI expression on mDCs was detected by flow cytometry. CD81 and SR-BI expression is shown as histograms (open curve) with isotype (solid curve). The results of one of three experiments are shown for each marker. (B) Anti-CD81 and anti-SR-BI inhibited HCV JFH1 infection of Huh7.5 cells. Huh7.5 cells were preincubated with anti-CD81 or anti-SR-BI for 1 h and infected with HCV JFH1 for 3 days. Infected Huh7.5 cells were stained for HCV core protein expression and counted by immunofluorescence. The proportion of Huh7.5 cells infected is represented on the y axis. (C) Anti-CD81 blocked HCV JFH1-induced mDC maturation. Purified mDCs were preincubated with anti-CD81 or anti-SR-BI for 1 h and cultured with HCV JFH1 at an MOI of 10 for 20 h. Maturation marker expression was evaluated by flow cytometry. The data are shown as the means and standard deviations of five healthy controls. (D and E) Anti-CD81 and anti-IL-10 do not restore the inhibitory effect of HCV JFH1 on the TLR ligand-dependent mDC activation of naive CD4 T cells. Purified mDCs were preincubated with anti-CD81 (D), anti-SR-BI (data not shown), or anti-IL-10 (E) for 1 h; incubated with HCV JFH1 for 4 h; and cultured with naive allogeneic CD4 T cells for 72 h. IFN- γ production was detected by ELISPOT assay ($n = 7$). (F) Anti-E2 blocks HCV JFH1-induced mDC maturation. mDCs were preincubated with anti-E2 before culture with HCV JFH1. The expression results for CD83, CD86, and CD40 from one representative experiment are shown. Three separate experiments with three separate donors were performed with similar results.

FIG. 7. Virus replication is not necessary for an HCV JFH1 effect on DC maturation. (A and B) HCV JFH1 was irradiated with UV at 254 nm (at a distance of 6 cm for 1 min). Freshly purified mDCs (A) and pDCs (B) were cultured with HCV JFH1 or UV-treated HCV JFH1 at an MOI of 10 for 20 h. TLR7 ligand R848 was added to pDC culture. Maturation marker was detected by flow cytometry. mDCs, $n = 4$; pDCs, $n = 7$.

MoDCs), different culture conditions including DC-activating stimuli [poly(I:C) versus lipopolysaccharide], or different amounts of virus used in these experiments. Regarding the latter issue, two doses of HCV JFH1 (MOIs of 10 and 1) were used in our experiments. This is equivalent to 1.5×10^8 and 1.5×10^7 RNA copies/ml or to 5×10^3 and 5×10^2 RNA copies/cell, respectively. Importantly, we found HCV JFH1 affected mDC maturation and T-cell activation at an MOI of 10 but not at an MOI of 1, which is actually in direct agreement with the results of Ebihara et al., who found no induction or inhibition of MoDC maturation at an equivalent RNA level to our MOI of 1 (18). Similarly, our results at 1.5×10^7 RNA copies/ml (MOI = 1) are also in agreement with the findings of Shiina et al. at 1×10^7 or 5×10^7 RNA copies/ml (equal to 50 or 100 copies/cell, respectively), which showed no effect on either mDC or MoDC maturation (48). Therefore, when the virus amount is increased to 1.5×10^8 RNA copies/ml or an

MOI of 10, as used here, the presence of HCV JFH1 stimulated mDC maturation. We propose that the virus particle concentration is a key factor for this effect on mDC maturation and function and that, while this concentration is probably 10-fold greater than average measurable plasma levels of HCV, there are likely compartments such as the liver where much higher virus particle concentrations exist, perhaps accounting for the mDC activation phenotype observed here and in our previous study (54) by mechanisms described here. Taken together, HCV JFH1 can activate mDCs in a virus concentration-dependent fashion.

CD81 is known to be involved in HCV entry and is known to interact with HCV E2 (11, 30, 43). Our data indicate that although virus replication was not necessary for HCV JFH1-induced mDC maturation (Fig. 7A), CD81-E2 interaction is necessary, and TLR2 is partially required (Fig. 5B, 6C, and 6F). We therefore propose that the interaction between HCV

E2 and CD81 is one step involved in HCV JFH1-mediated mDC activation. Consistent with this model is the prior finding that that HCV E2 increases MoDC CD80, CD83, CD86, CD40, and major histocompatibility complex II expression (57). However, in contrast to results obtained by Zhou et al. (57) that E2-matured MoDCs showed a greater capacity to stimulate T-cell proliferation, IFN- γ production, and IL-12 production, we found an impaired mDC capacity to activate naive T cells. One likely factor in the differences observed between that study and the results presented here is that intact virus particles were used here. In fact, as stated above, we also see a partial dependence on TLR2 and an overlapping profile of effects conferred by recombinant HCV core protein, suggesting that there is a mechanism beyond just interaction with CD81, likely in part related to HCV core protein interacting with TLR2, that accounts for a different activation phenotype and function.

HCV core protein has been previously reported to suppress DC function (16, 46). We found that HCV core protein exhibited similar effects compared to HCV JFH1 on DCs, except for inhibiting TLR ligand-induced mDC IL-6 production and pDC IFN- α production. Notably, the HCV core protein concentration used in these *in vitro* cultures (10 μ g/ml) is well above that which exists in the periphery of infected humans (100 pg/ml). The final HCV core concentration within HCV JFH1 culture experiments was 3 μ M or 66 ng/ml and is also above that which normally exists in the periphery of infected humans. Again, as stated above, within local regions of infected liver the concentration of HCV may exceed that which exists in the periphery and perhaps reach the levels used here. These results suggest that HCV core may mediate at least a portion of activities induced by HCV JFH1 and that HCV core protein free from intact virus particles present in virus preparations, such as are present in human plasma (1, 9, 40), may mediate a portion of this effect. TLR2 present on innate immune cells has been previously reported to interact with HCV core and NS3 proteins (49). Our results with TLR2 blockade suggest that this mechanism may be operative in direct virus particle-DC interactions (Fig. 5B).

TLR ligand-dependent mDC activation of naive CD4 T cells was found to be impaired by HCV JFH1, an effect apparently in opposition to what would be expected based upon the enhanced maturation phenotype observed with direct stimulation of mDC with HCV JFH1. This suggests that the mechanism of HCV JFH1 impairment of mDC activation of naive CD4 cells may be different from that of HCV JFH1-induced mDC maturation. Consistent with the concept of differing mechanisms, HCV JFH1-mediated inhibition of mDC activation of naive T cells was not abrogated by CD81 or TLR2 blockade. While T-cell activation and DC activation assays differed in time course (72 h versus 20 h), it is reasonable to consider that different mechanisms are likely operative. While IL-10 is known in other systems (including HCV systems) to be operative in the inhibition of T-cell activation and Th1 polarization, we found no evidence for this factor participating in the HCV JFH1 effects observed in our system (15, 45).

We and others have previously reported pDC numerical and functional defects in chronic HCV infection (2, 49, 54). In the present study, we found that HCV JFH1 inhibited TLR7 ligand-mediated pDC CD40 expression, and this was associated

with a decreased ability to activate naive CD4 T cells, indicating that reduced CD40 expression may play a role in reduced T-cell activation. The latter would be entirely consistent with the known role of CD40-CD40L interaction in the pDC activation of T cells toward Th1 polarization (10). Neither of these effects appeared to be mediated by CD81, and virus replication was not required. To our knowledge, this is the first report that *in vitro*-cultured HCV affects TLR ligand-induced pDC maturation marker expression and naive T-cell activation. Interestingly, although HCV JFH1 increased TLR7 ligand-induced pDC TNF- α production, we found that HCV JFH1 had no effect on TLR7-induced pDC IFN- α production (Fig. 2B). Shiina and Rehmann (48) recently reported that HCV JFH1 inhibits CpG-induced pDC IFN- α . In additional assays (not shown) we also find that HCV JFH1 inhibits CpG induced pDC IFN- α and TNF- α production, suggesting that HCV JFH1 has different effects on different TLR ligand-induced pDC activities.

In conclusion, our studies demonstrate that HCV JFH1-stimulated mDC maturation is mediated by CD81 and partially by TLR2, whereas HCV JFH1-inhibited TLR7 ligand-induced pDC CD40 expression is independent of CD81. The effects of HCV JFH1 on DCs did not require virus replication. Moreover, HCV JFH1 impaired the naive T-cell activating capacity of both mDCs and pDCs. By using recombinant HCV proteins, we further demonstrated that HCV core protein may mediate a portion of the HCV JFH1 effect on DC maturation and naive CD4 T-cell activation capacity. These results indicate differential mechanisms of effects of HCV on mDC and pDC function in HCV-infected individuals and provide targets for future mechanistic and interventional studies.

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REFERENCES

- Andre, P., G. Perlemtuer, A. Budkowska, C. Brechot, and V. Lotteau. 2005. Hepatitis C virus particles and lipoprotein metabolism. *Semin. Liver Dis.* 25:93–104.
- Anthony, D. D., N. L. Yonkers, A. B. Post, R. Asaad, F. P. Heinzl, M. M. Lederman, P. V. Lehmann, and H. Valdez. 2004. Selective impairments in dendritic cell-associated function distinguish hepatitis C virus and HIV infection. *J. Immunol.* 172:4907–4916.
- Auffermann-Gretzinger, S., E. B. Keefe, and S. Levy. 2001. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. *Blood* 97:3171–3176.
- Bain, C., A. Fatmi, F. Zoulim, J. P. Zarski, C. Trepo, and G. Inchauspe. 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 120:512–524.
- Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18:767–811.
- Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245–252.
- Barth, H., E. K. Schnober, C. Neumann-Haefelin, C. Thumann, M. B. Zeisel, H. M. Diepolder, Z. Hu, T. J. Liang, H. E. Blum, R. Thimme, M. Lambotin, and T. F. Baumert. 2008. Scavenger receptor class B is required for hepatitis C virus uptake and cross-presentation by human dendritic cells. *J. Virol.* 82:3466–3479.
- Blackard, J. T., L. Smeaton, Y. Hiasa, N. Horiike, M. Onji, D. J. Jamieson, I. Rodriguez, K. H. Mayer, and R. T. Chung. 2005. Detection of hepatitis C virus (HCV) in serum and peripheral-blood mononuclear cells from HCV-

- monoinfected and HIV/HCV-coinfected persons. *J. Infect. Dis.* **192**:258–265.
9. Bouvier-Alias, M., K. Patel, H. Dahari, S. Beaucourt, P. Larderie, L. Blatt, C. Hezode, G. Picchio, D. Dhumeaux, A. U. Neumann, J. G. McHutchison, and J. M. Pawlotsky. 2002. Clinical utility of total HCV core antigen quantification: a new indirect marker of HCV replication. *Hepatology* **36**:211–218.
 10. Cella, M., F. Facchetti, A. Lanzavecchia, and M. Colonna. 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat. Immunol.* **1**:305–310.
 11. Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic. 2004. CD81 is an entry coreceptor for hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **101**:7270–7274.
 12. Cox, A. L., T. Mosbrugger, Q. Mao, Z. Liu, X. H. Wang, H. C. Yang, J. Sidney, A. Sette, D. Pardoll, D. L. Thomas, and S. C. Ray. 2005. Cellular immune selection with hepatitis C virus persistence in humans. *J. Exp. Med.* **201**:1741–1752.
 13. Craxi, A., G. Laffi, and A. L. Zignego. 2008. Hepatitis C virus (HCV) infection: a systemic disease. *Mol. Aspects Med.* **29**:85–95.
 14. Decalf, J., S. Fernandes, R. Longman, M. Ahloulay, F. Audat, F. Lefrerre, C. M. Rice, S. Pol, and M. L. Albert. 2007. Plasmacytoid dendritic cells initiate a complex chemokine and cytokine network and are a viable drug target in chronic HCV patients. *J. Exp. Med.* **204**:2423–2437.
 15. de Jong, E. C., H. H. Smits, and M. L. Kapsenberg. 2005. Dendritic cell-mediated T-cell polarization. *Springer Semin. Immunopathol.* **26**:289–307.
 16. Dolganiuc, A., K. Kodys, A. Kopasz, C. Marshall, T. Do, L. Romics, Jr., P. Mandrek, M. Zapp, and G. Szabo. 2003. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J. Immunol.* **170**:5615–5624.
 17. Ebihara, T., M. Shingai, M. Matsumoto, T. Wakita, and T. Seya. 2008. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. *Hepatology* **48**:48–58.
 18. Ebihara, T., M. Shingai, M. Matsumoto, T. Wakita, and T. Seya. 2008. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. *Hepatology* **48**:48–58.
 19. Freeman, A. J., G. J. Dore, M. G. Law, M. Thorpe, J. Von Overbeck, A. R. Lloyd, G. Marinos, and J. M. Kaldor. 2001. Estimating progression to cirrhosis in chronic hepatitis C virus infection. *Hepatology* **34**:809–816.
 20. Gelderblom, H. C., L. E. Nijhuis, E. C. de Jong, A. A. te Velde, D. Pajkrt, H. W. Reesink, M. G. Beld, S. J. van Deventer, and P. L. Jansen. 2007. Monocyte-derived dendritic cells from chronic HCV patients are not infected but show an immature phenotype and aberrant cytokine profile. *Liver Int.* **27**:944–953.
 21. Gilliet, M., W. Cao, and Y. J. Liu. 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat. Rev. Immunol.* **8**:594–606.
 22. Greenbaum, E., R. Nir-Paz, D. M. Linton, T. Ben-Hur, A. Meirovitz, and Z. Zakay-Rones. 2004. Severe influenza infection in a chronic hepatitis C carrier: failure of protective serum HI antibodies after IM vaccination. *J. Clin. Virol.* **29**:23–26.
 23. Horlock, C., F. Shakib, J. Mahdavi, N. S. Jones, H. F. Sewell, and A. M. Ghaemmaghami. 2007. Analysis of proteomic profiles and functional properties of human peripheral blood myeloid dendritic cells, monocyte-derived dendritic cells and the dendritic cell-like KG-1 cells reveals distinct characteristics. *Genome Biol.* **8**:R30.
 24. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* **168**:4531–4537.
 25. Ito, T., R. Amakawa, T. Kaisho, H. Hemmi, K. Tajima, K. Uehira, Y. Ozaki, H. Tomizawa, S. Akira, and S. Fukuhara. 2002. Interferon- α and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. *J. Exp. Med.* **195**:1507–1512.
 26. Jarrossay, D., G. Napolitani, M. Colonna, F. Sallusto, and A. Lanzavecchia. 2001. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur. J. Immunol.* **31**:3388–3393.
 27. Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* **194**:863–869.
 28. Kallinowski, B., W. Jilg, L. Buchholz, W. Stremmel, and S. Engler. 2003. Immunogenicity of an accelerated vaccination regime with a combined hepatitis A/B vaccine in patients with chronic hepatitis C. *Z. Gastroenterol.* **41**:983–990.
 29. Kanto, T., N. Hayashi, T. Takehara, T. Tatsumi, N. Kuzushita, A. Ito, Y. Sasaki, A. Kasahara, and M. Hori. 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J. Immunol.* **162**:5584–5591.
 30. Kapadia, S. B., H. Barth, T. Baumert, J. A. McKeating, and F. V. Chisari. 2007. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. *J. Virol.* **81**:374–383.
 31. Krug, A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur. J. Immunol.* **31**:3026–3037.
 32. Lauer, G. M., and B. D. Walker. 2001. Hepatitis C virus infection. *N. Engl. J. Med.* **345**:41–52.
 33. Lechner, F., N. H. Gruener, S. Urbani, J. Uggeri, T. Santantonio, A. R. Kammer, A. Cerny, R. Phillips, C. Ferrari, G. R. Pape, and P. Klennerman. 2000. CD8⁺ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur. J. Immunol.* **30**:2479–2487.
 34. Lindenschmidt, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice. 2005. Complete replication of hepatitis C virus in cell culture. *Science* **309**:623–626.
 35. Liu, Y. J. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **106**:259–262.
 36. Liu, Y. J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* **23**:275–306.
 37. Logvinoff, C., M. E. Major, D. Oldach, S. Heyward, A. Talal, P. Balfe, S. M. Feinstone, H. Alter, C. M. Rice, and J. A. McKeating. 2004. Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc. Natl. Acad. Sci. USA* **101**:10149–10154.
 38. Longman, R. S., A. H. Talal, I. M. Jacobson, M. L. Albert, and C. M. Rice. 2004. Presence of functional dendritic cells in patients chronically infected with hepatitis C virus. *Blood* **103**:1026–1029.
 39. Longman, R. S., A. H. Talal, I. M. Jacobson, C. M. Rice, and M. L. Albert. 2005. Normal functional capacity in circulating myeloid and plasmacytoid dendritic cells in patients with chronic hepatitis C. *J. Infect. Dis.* **192**:497–503.
 40. Maillard, P., K. Krawczynski, J. Nitkiewicz, C. Bronnert, M. Sidorkiewicz, P. Gounon, J. Dubuisson, G. Faure, R. Crainic, and A. Budkowska. 2001. Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. *J. Virol.* **75**:8240–8250.
 41. Navas, M. C., A. Fuchs, E. Schvoerer, A. Bohbot, A. M. Aubertin, and F. Stoll-Keller. 2002. Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. *J. Med. Virol.* **67**:152–161.
 42. Piccoli, D., S. Tavarini, S. Nuti, P. Colombatto, M. Brunetto, F. Bonino, P. Ciccorossi, F. Zorat, G. Pozzato, C. Comar, S. Abrignani, and A. Wack. 2005. Comparable functions of plasmacytoid and monocyte-derived dendritic cells in chronic hepatitis C patients and healthy donors. *J. Hepatol.* **42**:61–67.
 43. Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani. 1998. Binding of hepatitis C virus to CD81. *Science* **282**:938–941.
 44. Russell, R. S., J. C. Meunier, S. Takikawa, K. Faulk, R. E. Engle, J. Bukh, R. H. Purcell, and S. U. Emerson. 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **105**:4370–4375.
 45. Saito, K., M. Ait-Goughoulte, S. M. Truscott, K. Meyer, A. Blazevic, G. Abate, R. B. Ray, D. F. Hoft, and R. Ray. 2008. Hepatitis C virus inhibits cell surface expression of HLA-DR, prevents dendritic cell maturation, and induces interleukin-10 production. *J. Virol.* **82**:3320–3328.
 46. Sarobe, P., J. J. Lasarte, N. Casares, A. Lopez-Diaz de Cerio, E. Baixeras, P. Labarga, N. Garcia, F. Borrás-Cuesta, and J. Pierto. 2002. Abnormal priming of CD4⁺ T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. *J. Virol.* **76**:5062–5070.
 47. Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.* **21**:5017–5025.
 48. Shiina, M., and B. Rehmann. 2008. Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. *Hepatology* **47**:385–395.
 49. Szabo, G., and A. Dolganiuc. 2005. Subversion of plasmacytoid and myeloid dendritic cell functions in chronic HCV infection. *Immunobiology* **210**:237–247.
 50. Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**:791–796.
 51. Wertheimer, A. M., A. Bakke, and H. R. Rosen. 2004. Direct enumeration and functional assessment of circulating dendritic cells in patients with liver disease. *Hepatology* **40**:335–345.
 52. Wiedmann, M., U. G. Liebert, U. Oesen, H. Porst, M. Wiese, S. Schroeder, U. Halm, J. Mossner, and F. Berr. 2000. Decreased immunogenicity of recombinant hepatitis B vaccine in chronic hepatitis C. *Hepatology* **31**:230–234.
 53. Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon. 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc. Natl. Acad. Sci. USA* **103**:2310–2315.

54. **Yonkers, N. L., B. Rodriguez, K. A. Milkovich, R. Asaad, M. M. Lederman, P. S. Heeger, and D. D. Anthony.** 2007. TLR ligand-dependent activation of naive CD4 T cells by plasmacytoid dendritic cells is impaired in hepatitis C virus infection. *J. Immunol.* **178**:4436–4444.
55. **Zeisel, M. B., G. Koutsoudakis, E. K. Schnober, A. Haberstroh, H. E. Blum, F. L. Cosset, T. Wakita, D. Jaeck, M. Doffoel, C. Royer, E. Soulier, E. Schvoerer, C. Schuster, F. Stoll-Keller, R. Bartenschlager, T. Pietschmann, H. Barth, and T. F. Baumert.** 2007. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. *Hepatology* **46**:1722–1731.
56. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* **102**:9294–9299.
57. **Zhou, Y., Y. Lukes, J. Anderson, B. Fileta, B. Reinhardt, and M. Sjogren.** 2007. Hepatitis C virus E2 envelope protein induces dendritic cell maturation. *J. Viral Hepat.* **14**:849–858.