Hepatitis C Virus (HCV) Core Protein-Induced, Monocyte-Mediated Mechanisms of Reduced IFN-α and Plasmacytoid Dendritic Cell Loss in Chronic HCV Infection

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IFN-α production by plasmacytoid dendritic cells (PDCs) is critical in antiviral immunity. In the present study, we evaluated the IFN-α-producing capacity of PDCs of patients with chronic hepatitis C virus (HCV) infection in treatment-naive, sustained responder, and nonresponder patients. IFN-α production was tested in PBMCs or isolated PDCs after TLR9 stimulation. Treatment-naive patients with chronic HCV infection had reduced frequency of circulating PDCs due to increased apoptosis and showed diminished IFN-α production after stimulation with TLR9 ligands. These PDC defects correlated with the presence of HCV and were in contrast with normal PDC functions of sustained responders. HCV core protein, which was detectable in the plasma of infected patients, reduced TLR9-triggered IFN-α and increased TNF-α and IL-10 production in PBMCs but not in isolated PDCs, suggesting HCV core induced PDC defects. Indeed, addition of TNF-α and IL-10 induced apoptosis and inhibited IFN-α production in PDCs. Neutralization of TNF-α and/or IL-10 prevented HCV core-induced inhibition of IFN-α production. We identified CD14+ monocytes as the source of TNF-α and IL-10 in the HCV core-induced inhibition of PDC IFN-α production. Anti-TLR2-, not anti-TLR4-, blocking Ab prevented the HCV core-induced inhibition of IFN-α production. Our findings reveal a novel HCV-driven mechanism for PDC loss that may provide an escape mechanism for HCV from immune surveillance.

Materials and Methods

Blood donors and cells

Healthy individuals (controls, n = 46), treatment-naive patients with chronic infection (HCV patients, n = 41), patients who cleared HCV (sustained responders, n = 16), and those who failed to clear HCV (nonresponders, n = 14) after therapy were enrolled in the study (Table I). Of responders, 12 were treated with Pegasus plus ribavirin for 24–48 mo, 1 received Pegasus plus ribavirin plus viramidin for 36 mo, 2 had polyethylene glycol-interon plus ribavirin for 48 mo, and 1 was treated with interferon15 plus ribavirin for 62 mo. The responders were enrolled in the study 9 mo to 4 years after the last dose of therapeutic agent and all have been HCV-free since. Nonresponders were previously on similar treatment regimens and did not clear the virus upon completion of the treatment; they were enrolled in the study at least 6 mo after the therapy was completed and had detectable viral counts upon recruitment into the study. A cohort of HCV-naive patients with nonalcoholic liver disease (nonalcoholic steatohepatitis (NASH)) with features of liver inflammation (elevated liver enzymes (alanine aminotransferase), lack of viral infection, and liver biopsy-proven inflammation) was recruited (n = 6). The study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts, and informed consent was obtained.

PBMcs were separated from peripheral blood using centrifugation in Ficoll-Paque Plus gradient (Amersham Biosciences), as described previously (12). The cells were cultured at 1 × 10⁶/ml in 96-well plates in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured for 7 days at 37°C with 5% CO₂. The media were changed every 2–3 days.
in RPMI 1640 medium (Invitrogen Life Technologies) with 10% FBS and 1% nonessential amino acids (Sigma-Aldrich). ODN2216 (CpG-A) was from InvivoGen; herpes simplex virus (HSV KOS-1 strain, UV-irradiated) was a gift from Dr. R. Welsh at the University of Massachusetts (Worcester, MA); rIL-10 and TNF-α were both from PeproTech. ELISA kits (IL-10 and TNF-α) were from BD Bioscience and IFN-α from BioSource International. HCV core protein was purchased from BioDesign. rHCV core protein and core protein contamination of recombinant proteins was determined by 0.01 EU/ml (by Limulus amebocyte assay; BioWhittaker). Blocking anti-TNF-α, isotype controls, and anti-TL2R and anti-TLR4 Abs were purchased from eBioscience; anti-II-10 Ab was from BioSource International.

PDCs were isolated from PBMCs using anti-BDCA2 Abs, according to the manufacturer’s recommendations (Miltenyi Biotec). Briefly, PBMCs (10^6 cells/100 µl of separation buffer (ice-cold PBS, 0.5% BSA, and 2 mM EDTA)) were incubated with FcR-blocking reagent (50 µl/10^6 cells) and anti-BDCA-2-biotin Ab (100 µl/10^6 cells) for 10 min at +4°C, followed by addition of 400 µl of separation buffer/10^6 cells, FcR-blocking reagent (150 µl/10^6 cells) and anti-biotin MicroBeads (200 µl/10^6 cells) for 15 min at +4°C. After incubation, PBMCs were washed with 20 volumes of cold separation buffer and filtered through a magnetic column. To increase the purity of BDCA2+ PDCs, the magnetic separation was repeated using a fresh column. The purity of the isolated PDCs, determined by flow cytometry analysis after staining with streptavidin-PE and anti-CD123-allophycocyanin Abs, was 95% (data not shown). For IFN-α production and apoptosis studies, PDCs were cultivated for 48 h in RPMI 1640 medium with 10% FBS and 5 ng/ml rIL-3 (PeproTech) in the absence or presence of TLR9 ligands.

CD14+ cells were separated from PBMCs using anti-CD14 Abs-coated magnetic beads (Miltenyi Biotec), as manufacturer recommended. Briefly, 10^6 PBMCs/80 µl were incubated with 20 µl of anti-CD14 Abs-coated microbeads for 15 min at +4°C, then washed with 20 volumes of separation buffer and purified as described for PDCs.

T lymphocytes were purified using the “T cell-negative isolation kit” (Dynal Biotech), according to manufacturer instructions. Briefly, PBMCs (1 × 10^7/200 µl of 10% FBS-PBS) were incubated with 20 µl of Ab mix (containing anti-CD14, CD16, CD56, HLA-DR/DP, and CD235a) for 10 min at 4°C, washed, and incubated with 100 µl of depletion Dynabeads for 15 min at room temperature. Non-T cells were separated in magnetic field, whereas T cells were washed and used for coculture with PDCs.

### Flow cytometry analysis

For phenotyping, PBMCs were stained with a labeled anti-lineage Abs (CD3, CD8, CD11c, CD14, CD20, and CD56) and anti-CD4, or anti-CD123 (all from BD Biosciences) and anti-BDCA-2 (Miltenyi Biotec), or matching isotype control Abs (BD Biosciences and Miltenyi Biotec), fixed with 2% paraformaldehyde in PBS, and analyzed by flow cytometry. One hundred thousand events from the gate corresponding to mononuclear cells were collected using a FACS Calibur flow cytometer and analyzed with CellQuest software (BD Biosciences). PDC apoptosis was detected using the ApoTarget kit (BioSource International) and analyzed by flow cytometry. PDCs were gated based on size and granularity and analyzed for the presence of fluorescent cells.

### RNA isolation and real-time PCR

Total RNA was isolated from frozen liver tissue using the RNeasy Micro-Kit from Qiagen, according to the manufacturer’s instructions. Reverse transcriptions were performed using the First-Strand cDNA Synthesis kit (Promega), according to manufacturer’s instructions. One microgram of total RNA was transcribed to cDNA in 20-µl reaction volume. For transcript quantification purposes by real-time PCR, the SYBR Green Mix containing Thermo-Start DNA Polymerase was used according to the manufacturer’s instructions (Eurogentec). Primers for CD123 (forward 5'−GAAGAAACCGTGTTTAACAC and reverse 5'−TAGCTTGAGTGATTCG) and BDCA-2 (forward 5'−TGGAAAGAAAGCACCCC and reverse 5'−TAGTCTTCTACAAGCGG) were from IDT. The 18S primers were purchased from Ambion. The PCR using 1 µl of cDNA was conducted in iCycler Thermal Cycler (Bio-Rad). A hot-start phase was applied at 95°C for 10 min for all primers. cDNA was amplified (45 cycles for CD123 and BDCA-2; 32 cycles for 18S) at 95°C for 10 s, 60°C for 10 s, and 72°C for 2 s. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by dsDNA-binding SYBR Green. A dissociation/melting curve of the PCR product was constructed in the range of 55°C to 95°C. Data were analyzed using the Bio-Rad iCycler software and comparative threshold (Ct) method with the following formula: \(ΔCt = Ct(\text{target}) - Ct(18S)\), where Ct is the cycle at which fluorescence is first detected above the baseline of each sample. The fold increase in the expression of CD123 and BDCA-2 mRNA in experimental groups compared with medium control was calculated as 2^(-ΔΔCt).
Statistical analysis
The Wilcoxon nonparametric test, Bartlett’s test, and Correlation Z score analyses in StatView (SSS Institute) program on a MacG4 computer (Apple) were used.

Results

Low IFN-α production correlates with reduced frequency of PDCs in the peripheral blood of patients with chronic HCV infection

IFN-α is a naturally occurring protein with distinct antiviral and immunoregulatory effects (13). Healthy individuals produce IFN-α in response to infection and stimulation with an Ag or mitogen. We tested the hypothesis that PBMCs from patients with chronic HCV infection may have impaired capacity to produce IFN-α. Baseline levels of IFN-α in PBMCs from treatment-naive chronic HCV-infected patients and normal controls were low and comparable (Fig. 1A). Because of the unique expression of TLR9 and the capacity of PDCs to produce IFN-α, evaluation of IFN-α levels after stimulation of PBMCs with TLR9 ligands, CpG-A or HSV, is reflective of PDC functions (5–7, 14, 15). Upon in vitro stimulation with TLR9 ligands, we found significantly reduced production of IFN-α in HCV-infected patients compared with controls (CpG-A, \( p < 0.004 \); HSV, \( p < 0.003 \)) (Fig 1A). Such a profound defect in IFN-α production could be explained by either functional impairment or loss of PDCs. To identify the circulating PDC population, we took advantage of the distinct surface markers, BDCA-2 and CD123, expressed on PDCs (6–8). Using flow cytometry, we found that patients with chronic HCV infection had a remarkable loss of the circulating population of PDCs (determined as BDCA2\(^{+}/\)CD123\(^{+}\)), compared with controls (Fig 1B). The average frequency of circulating PDCs in HCV-infected patients was reduced to 0.12 ± 0.04%, from an average of 0.28 ± 0.11% seen in controls (\( p < 0.0003 \)) (Fig. 1C).

Increased PDC apoptosis and impaired IFN-α production correlate with active HCV infection

Cell death by apoptosis, characterized by phosphatidylserine exposure on integral cellular membranes, or necrosis may account for the loss of a cell population (16). Annexin V\(^{FITC}/\)propidium iodide (PI) staining revealed that PDCs isolated from healthy controls (data not shown), suggesting that cellular necrosis is unlikely to account for the loss of circulating PDCs. Overall, there was a significantly higher apoptosis in the PDCs of HCV patients compared with controls both at baseline (\( p < 0.026 \)) and after TLR9 stimulation (\( p < 0.032 \)) (Fig. 2B). These data suggested that PDCs from HCV patients were in vivo programmed for apoptosis.

To assess the functional capacity of PDCs, we purified BDCA2\(^{+}\) cells using magnetic cell sorting. Although the recovery of PDCs from PBMCs was proportional to the low frequency of PDCs determined by flow cytometry, there were no differences in the levels of expression of CD123 and BDCA2 markers between PDCs isolated from HCV patients and controls (data not shown). Equal numbers of PDCs purified from HCV patients produced significantly lower levels of IFN-α in vitro stimulation with TLR9 ligands compared with controls (Fig. 2C). These results led us to the conclusion that there were two components to the reduced IFN-α production by PDCs seen in HCV-infected patients: first, the frequency of circulating PDCs was reduced, and second, there was an additional defect in the functional capacity of PDCs to produce IFN-α.

To determine whether PDC dysfunction correlated with ongoing HCV infection, we tested patients who cleared the HCV virus after IFN-α-based therapy and had no detectable viral levels (sustained responders) and compared with those who failed to clear HCV after therapy (nonresponders). In contrast to nonresponder patients with detectable HCV load, sustained responders showed no difference in IFN-α production compared with HCV-naive controls (Fig. 3A). In addition, the frequency of circulating PDCs was comparable between sustained responders (0.3 ± 0.22%) and controls (0.28 ± 0.15%) but was reduced in nonresponders (0.19 ± 0.09%) (Fig. 3B). We identified that the PDC frequency inversely correlated with the levels of HCV core protein in the plasma of patients with chronic HCV infection (Fig. 3C) and did not correlate with the patients’ age, viral count, or liver enzymes (data not shown). These results suggested that ongoing viral replication and/or virus-induced mechanisms may be responsible for the loss of PDCs in patients.

HCV core protein inhibits IFN-α production in PBMCs but not in isolated PDCs

Based on the observation that PDC loss was associated with ongoing HCV infection and the presence of detectable HCV core
protein in chronically infected patients, we focused on viral factors that may account for the PDC defects. Among the 10 different proteins encoded by the HCV genome, core protein has multiple immunomodulatory effects (17). Furthermore, HCV core protein is detectable in the peripheral blood (Fig. 3), allowing it to interact with immune cells outside of the liver (18). Consequently, we

![FIGURE 2](image-url)

**FIGURE 2.** Purified PDCs from HCV-infected patients are apoptotic and produce low IFN-α. PDCs were purified by MACS, based on BDCA-2 expression, from normal controls and treatment-naive HCV patients. A, Freshly isolated PDCs or PDCs stimulated with TLR9 ligand (UV-irradiated HSV, 1 PFU/cell) for 48 h were stained with Annexin V FITC and PI and analyzed by flow cytometry. Upper left quadrant represents necrotic cells, upper right represents late apoptosis, lower right represents early apoptosis, and lower left are viable cells. The fluorescence intensity dot plots are shown from one representative control (top) and one patient with chronic HCV infection (bottom). B, PDCs were cultivated, stained, and analyzed as in A. Data are shown as the frequency of annexin V+ cells from PDCs of 14 normal and 18 HCV-infected individuals (+ indicates p < 0.05 between medium and HSV-stimulated (TLR9) samples in controls; # indicates p < 0.05 between medium and TLR9-stimulated samples in HCV patients). C, PDC from controls and HCV patients (1 × 10⁵/well) were stimulated in vitro with CpG-A (5 mM) or UV-irradiated HSV (1 PFU/cell) for 48 h, and IFN-α production was analyzed by ELISA (+ indicates p < 0.005 between medium and CpG-A and p < 0.001 between medium and HSV in controls; # indicates p < 0.025 between medium and CpG-A and p < 0.032 between medium and HSV in HCV patients).

**FIGURE 3.** IFN-α production and frequency of PDCs is normalized in patients who cleared HCV infection after antiviral therapy. PBMC (1 × 10⁷/ml) of controls (n = 26) and HCV patients who cleared (sustained responders, n = 16) or failed to clear the virus after therapy (nonresponders, n = 14) were stimulated in vitro with TLR9 ligand (CpG-A, 5 mM) for 48 h to assess IFN-α production by ELISA (A) or stained with anti-CD123 and anti-BDCA-2 Abs and analyzed by flow cytometry (B). No significant changes between controls and responders were observed. C, The HCV core protein in plasma of HCV patients was quantified using Ortho HCV core Ag ELISA. The frequency of PDCs in peripheral circulation was plotted against the corresponding value of plasma HCV core protein. Correlation z score = -3.402, p < 0.026 (Bartlett test). The inset shows the magnified picture for the HCV core values >1000 fMol/L. 

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Hypothized that HCV core protein may impair IFN-α production. As shown in Fig. 4A, addition of rHCV core protein failed to induce IFN-α production or affect IFN-α production in TLR9-stimulated purified PDCs. In contrast, we found a significant reduction of TLR9-triggered IFN-α production in PBMCs in the presence of HCV core protein (Fig. 4B). Our recombinant core protein contained β-galactosidase as a stabilizer; thus, to control for the biological activity of the core protein, we included a β-galactosidase control in the assay. As shown in Fig. 4B, β-galactosidase did not activate PBMCs and failed to influence TLR9-triggered IFN-α production. This suggested the possibility that HCV core protein indirectly inhibited IFN-α production by PDCs and perhaps affected a cell population other than PDCs.

PDC function is regulated closely by the cytokine microenvironment (19); thus, we sought to evaluate whether HCV core protein-induced changes in the cytokine milieu of TLR9-stimulated PBMCs affected IFN-α production. We have shown previously that HCV core protein triggers cytokine production in monocytes (12, 20). Consistent with this, HCV core protein, alone or in combination with a TLR9 ligand, induced IL-10 (Fig. 4C) and TNF-α (Fig. 4D) in PBMCs from controls and from HCV-infected patients. TLR9 stimulation alone induced minimal IL-10 or TNF-α compared with unstimulated cells and did not alter HCV core-induced production of these cytokines. The levels of IL-10 and TNF-α were consistently higher in HCV-infected patients compared with controls. Consistent with our previous study (12, 20), the β-galactosidase control did not induce IL-10 or TNF-α in monocytes (Fig. 4, C and D). Taken together, these data implied that in the PBMC population, HCV core protein did not affect PDCs directly; however, it triggered production of both IL-10 and TNF-α that, in turn, may affect IFN-α production by PDCs.

IL-10 and TNF-α inhibit TLR9-induced IFN-α production and induce PDC apoptosis

To investigate the possibility that HCV core protein-induced IL-10 and/or TNF-α could influence IFN-α production, we stimulated PBMCs or PDCs with rIL-10 or TNF-α in the presence or absence of TLR9 stimulation. Addition of rIL-10 significantly inhibited TLR9-induced IFN-α production in both PBMCs (Fig. 5A) and PDCs (data not shown) in a dose-dependent manner. The lowest dose of IL-10 concentration (100 pg/ml) that was comparable to levels induced in PBMCs of HCV-infected patients (Fig. 4C) still resulted in inhibition of IFN-α production by PDCs. Flow cytometry analysis of Annexin VFITC/PI-stained cells revealed that rIL-10 alone induced apoptosis in PDCs, and an even more dramatic increase in PDC apoptosis was observed when rIL-10 was added together with a TLR9 ligand (Fig. 5B). Both the highest dose of IL-10 (10 ng/ml) and the combination of IL-10 with a TLR9 ligand resulted in an increase of PI−, late apoptotic cells.

Similar to the effect of IL-10, rTNF-α abolished TLR9-induced IFN-α production in a dose-dependent manner in PBMCs (Fig. 5C) and in PDCs (data not shown). TNF-α also induced PDC apoptosis and augmented TLR9-induced cell death (Fig. 5D).

These results demonstrated that IL-10 and TNF-α each inhibit IFN-α production and induce apoptosis of PDCs.

Monocytes are the source of HCV core-induced cytokines that inhibit IFN-α production by PDCs

Since rIL-10 and TNF-α could mimic the effects of HCV core protein on TLR9-induced IFN-α production in PBMCs, we investigated whether neutralization of IL-10 and TNF-α could eliminate the inhibitory effect of HCV core protein on IFN-α production. As shown in Fig. 6A, anti-IL-10- and anti-TNF-α-neutralizing Abs each partially blocked the inhibitory effect of HCV core protein on IFN-α production by PBMCs. Furthermore, the combination of
anti-IL-10- and anti-TNF-α-neutralizing Abs resulted in an additive effect and totally blocked IFN-α inhibition by HCV core protein. To confirm the specificity of the neutralizing Abs, we mimicked the HCV core action by adding rIL-10, TNF-α, and their combination to TLR9-stimulated PBMCs and demonstrated that anti-IL-10- and anti-TNF-α-neutralizing Abs could prevent the IL-10- and TNF-α-induced inhibition of IFN-α production, respectively. These data confirmed that both IL-10 and TNF-α are implicated in HCV core-mediated inhibition of IFN-α production.

PBMCs represent a heterogeneous population consisting of lymphocytes, NK cells, monocytes, and circulating DCs. Of those, lymphocytes produce negligible amounts of IL-10, whereas monocytes are the main producers of IL-10 and TNF-α due to their numeric prevalence over DCs. Thus, we tested the hypothesis that monocytes are the source of HCV core-induced IL-10 and TNF-α that inhibit IFN-α production by PDCs. Magnetic bead separation of CD14+ cells from PBMCs resulted in a 100% pure CD14+ population and a ≥90% pure CD14+ population (data not shown). Depletion of CD14+ cells did not affect TLR9-induced production of IFN-α, but it fully prevented the HCV core-induced inhibition of IFN-α production in TLR9 ligand-stimulated PBMCs (Fig. 6B).

The purified CD14+ cell population from both HCV-infected patients and normal controls produced IL-10 (Fig. 6C) and TNF-α (Fig. 6D) in response to HCV core and LPS, a TLR4/CD14 ligand, used as a positive control for monocyte activation. These results confirmed that CD14+ monocytes are the primary target of HCV core protein in induction of IL-10 and TNF-α that inhibit IFN-α production by PDCs. HCV core protein inhibited TLR9-triggered IFN-α production in a dose-dependent manner (Fig. 6E), and the lowest dose of HCV core protein that affected IFN-α (∼4500 fM) was close to the range of HCV core protein levels detected in plasma of patients with chronic HCV infection (Fig. 3C).

The critical role of monocytes in mediation of HCV core-induced inhibition of IFN-α production was further investigated in cocultures of isolated PDCs with monocytes or T lymphocytes. While TLR9 stimulation induced IFN-α production in PDCs in the presence of either monocytes or T cells, inhibition of IFN-α production by HCV core protein occurred only when PDCs were cocultured with monocytes and not with T cells (Fig. 7A). Both TNF-α and IL-10 were induced by HCV core protein in the monocyte-PDC but not in the T cell-PDC cocultures, further pointing to the role of monocyte-derived cytokines in inhibition of IFN-α production by PDCs (Fig. 7, B and C). We and others have reported previously that HCV core protein triggers monocyte activation via TLRs, in particular by membrane
expressed TLR2 (20) and TLR4 (21). In the present study, we found that anti-TLR2, but not anti-TLR4, can partially restore the HCV core-mediated inhibition of TLR9-triggered IFN-α production in PBMCs (Fig. 7D). The specific activity of the anti-TLR2- and anti-TLR4-blocking Abs was indicated by inhibition proteoglycan (TLR2 ligand)- and LPS (TLR4 ligand)-mediated TNF-α production in monocytes, respectively (Fig. 7E). These data suggest that TLR2 plays a critical role in monocyte activation and is implicated in the inhibitory effect of HCV core on IFN-α production by PDCs.

To examine whether HCV core protein and the cytokine milieu played a role in PDC functional impairment, we analyzed a cohort of patients with NASH, an inflammatory liver disease of nonviral etiology (22). We found that the frequency of PDCs in the peripheral circulation (Fig. 8A) and the production of IFN-α (Fig. 8B), TNF-α (Fig. 8C), and IL-10 (Fig. 8D) in PBMCs were comparable between patients with NASH and controls. Furthermore, similar to controls, HCV core protein reduced the TLR9-triggered IFN-α production in PBMCs of NASH patients (Fig. 8B), suggesting that HCV core protein can impair PDCs functions.

Finally, we considered the hypothesis that the apparent “loss” of PDC numbers in the blood of patients with chronic HCV infection could be due to PDC homing to the liver, the primary site of HCV infection. We identified that the levels of mRNA coding for the PDC markers (5–8), CD123 (Fig. 9A), and BDCA-2 (Fig. 9B), were significantly higher in the livers of patients with HCV infection compared with noninfected controls or patients with NASH, suggesting that chronic HCV infection may lead to PDC compartmentalization.

**Discussion**

Although various host- and virus-derived factors can account for viral persistence during the course of chronic hepatitis C infection, the complexity of the immune defects and the interactions between the virus and different immune cell types remains to be fully understood. In the present study, we report that patients with chronic HCV infection have reduced capacity to produce IFN-α after in vitro stimulation of PDCs. Our studies delineated at least three reasons for the reduced IFN-α production in these patients. First, we found a loss of circulating PDCs due to apoptosis. Second, there was reduced capacity of existing circulating PDCs to produce IFN-α. Third, we identified increased PDC homing to the liver in HCV infection. We also demonstrated that reduced circulating PDC frequency correlated with increased plasma levels of HCV core protein. Furthermore, we found that in PBMC HCV core protein triggered monocyte-derived IL-10 and TNF-α production. These cytokines, in turn, led to PDC apoptosis and impaired production of IFN-α, thus closely resembling the PDC defects seen in chronically HCV-infected patients.

PDCs produce large amounts of IFN-α upon viral infection (7, 8). We found that PDCs of HCV-infected patients had reduced IFN-α production capacity upon TLR9 stimulation with a synthetic ligand, CpG-A, or a natural ligand, UV-inactivated HSV (6–8, 14, 15). These data are in agreement with previous publications from Murakami et al. (23) and Wertheimer et al. (24), and in contradiction with a report from Longman et al. (25), possibly due to the differences in the approaches taken to evaluate the IFN-α-producing capacity of PDCs. IFN-α is detrimental for establishing an effective link between the innate and adaptive immunity via up-regulation of IFN-γ in CD4+ and CD8+ T cells and NK cells, modulation of T cell responsiveness to IL-12-induced secretion of IFN-γ, and subsequent drive of a Th1 response (7, 26). Indeed, multiple immune defects have been described in patients with
chronic HCV infection that may be linked to reduced IFN-\(\gamma\)/H\(_9251\) production, including insufficient response of CTLs, low activity of NK cells, and production of Abs with low neutralizing capacity (2, 13, 27–29). It has been reported that IFN-\(\gamma\)/H\(_9251\) is a potent survival factor of PDCs (30, 31); thus, internal deficit of IFN-\(\gamma\)/H\(_9251\) may favor a self-destructive loop for loss of PDCs in HCV-infected patients.

**FIGURE 7.** HCV core impairs TLR9-induced IFN-\(\gamma\) production in PDCs in the presence of monocytes but not T lymphocytes. BDCA-2\(^+\) PDCs from healthy donors (1 \(\times\) 10\(^5\)/well) and syngeneic monocytes (1 \(\times\) 10\(^6\)/well, □) or T lymphocytes (1 \(\times\) 10\(^5\)/well, ▬) were stimulated in vitro with TLR9 ligand (CpG-A, 5 mM) with or without HCV core protein (22,500 fM) for 48 h at 1:10 PDC:monocyte or PDC:T cell ratio. The production of IFN-\(\gamma\) (A), TNF-\(\gamma\) (B), and IL-10 (C) was analyzed in ELISA (\(n = 3\)). D, Human PBMCs were incubated with indicated Abs for 30 min at room temperature, then stimulated with TLR9 ligand (HSV, 1 PFU/cell) alone or with a combination of TLR9 ligand plus HCV core (22,500 fM). Culture supernatants were analyzed for IFN-\(\gamma\) (B), TNF-\(\gamma\) (C), or IL-10 (D) production in specific ELISA; data are shown as mean \(\pm\) SE ng/ml.

**FIGURE 8.** The frequency of PDCs and cytokine production in NASH patients is comparable to controls. A, The frequency of PDCs (BDCA2\(^+\)CD123\(^+\)) in individual controls and NASH patients is shown. The horizontal bars indicate the means; ns indicate no significant changes. B–D, PBMCs (1 \(\times\) 10\(^7\)/sample/ml) from normal controls (\(n = 4\)) or NASH patients (\(n = 6\)) were stimulated with TLR9 ligand (HSV, 1 PFU/cell) alone or with a combination of TLR9 ligand plus HCV core (22,500 fM). Culture supernatants were analyzed for IFN-\(\gamma\)-\(\alpha\) (B), TNF-\(\gamma\)-\(\alpha\) (C), or IL-10 (D) production in specific ELISA; data are shown as mean \(\pm\) SE ng/ml.
The proteins are produced. High IFN- 
ated antiviral effects would be most important in the liver, where (34). The functional capacity of those PDCs in the liver remains to DC morphology were identified in livers of HCV-infected patients the loss in virus-specific immune activation in chronic HCV infection. Reduced absolute numbers of the circulating PDCs were consistent with this notion, CD80
 cote on monocytes has independent recognition and stimulatory function, while in T cells, TLR2 requires TCR protein because TLR2 on monocytes has independent recognition and direct role for the virus in the loss and functional impairment of PDCs. HCV encodes a long polyprotein that is cleaved into at least 10 known proteins (37). Core protein, the first to be cleaved from the viral polyprotein, has a distinct immuno-regulatory capacity. In agreement with previous studies (18, 38), we found that HCV core protein was found in the peripheral blood of all analyzed HCV-infected patients. Furthermore, we identified an inverse relationship between plasma core protein levels and circulating PDC frequency in patients with chronic HCV infection. Our studies revealed that HCV core protein induced IL-10 and TNF- 
production in monocytes, and these cytokines in turn resulted in PDC apoptosis and impaired capacity of PDCs to produce IFN- 

HCV core protein can interact with multiple cellular factors; regulate expression of cellular genes and control signaling pathways in different cell types, including DCs (reviewed in Refs. 17 and 37). We have reported previously that monocytes recognize HCV core protein via TLR2 and TLR2 activation triggers intra-cellular signaling pathways to induce TNF- 
(20). Recently, TLR4 was also implicated in cellular activation by HCV core protein (21). In the present study, we found that anti-TLR2, but not anti-TLR4, Abs partially prevented the inhibitory effect of HCV core protein on TLR9-triggered IFN- 
production, thus suggesting that core protein, via TLR2, plays a role in modulation of PDC function. We have shown previously that the intracellular signaling pathways triggered by HCV core involved in activation of NF- 
, AP-1, and the MAPKs in induction of IL-10 and TNF- 
(20). Both IL-10 and TNF- 
production were induced in macrophages by HCV core protein at concentrations comparable to HCV core protein levels found in the sera of HCV-infected individuals. It is also likely that macrophages and monocytes in the liver may be exposed to even higher concentrations of HCV core protein than the circulating levels. Furthermore, monocytes of patients with chronic HCV infection produced higher levels IL-10 and TNF- 
, and increased levels of these cytokines were reported in plasma from HCV-infected patients compared with controls (39, 40). In the present study, we demonstrated that Ab neutralization of IL-10 and TNF- 
restored IFN- 
production in TLR9 plus HCV core-stimulated PBMCs. Furthermore, rIL-10 and TNF- 
absorbed both TLR9-induced IFN- 
production and PDC apoptosis in a dose-dependent manner. This observation is in agreement with previous publications showing that the microenvironment, including the presence of cytokines and chemokines, is crucial for PDC function (19, 41–43). ILs 3, 4, 7, and 15 promote IFN- 
production was affected only in PDC/monocyte but not in PDC/T cell cocultures. This may be related to TLR2 recognition of core protein because TLR2 on monocytes has independent recognition and stimulatory function, while in T cells, TLR2 requires TCR costimulation for cell activation (46, 47).

Under normal conditions, both TNF- 
and IL-10 limit overproduction of IFN- 
by activated PDCs during resolution of viral infections (19). Our data suggest that HCV can steal this protective mechanism to achieve a destructive effect on PDCs by inducing TNF- 
and IL-10 in monocytes. It has been suggested previously that IL-10 may reduce PDC viability, but the mechanism of IL-10-induced PDC death is unknown to date (41). We dissected the mechanism of IL-10- and TNF- 
-induced PDC death and found that both IL-10 and TNF- 
induced PDC apoptosis. Although this effect is expected for TNF- 
, a known trigger of apoptosis in different cell types, the observation of PDC apoptosis induction by IL-10 is novel. A recent report by Marra et al. (48) showed that IL-10 induces regulatory T cell apoptosis by up-regulation of the membrane form of TNF- 
. Consequently, it is not surprising that we found an additive protection by anti-IL-10 plus anti-TNF- 
Abs on PDC function.

We showed that chronic HCV infection induces cytokine changes and leads to PDC dysfunction as measured by IFN- 
production and apoptosis; thus, one could suspect that HCV may induce global immune suppression. In support of this notion, recent data from El-Sarag et al. (49) from a case-control study showed

FIGURE 9. HCV-infected patients have elevated levels of PDC markers CD123 and BDCA-2 in the liver. The levels of RNA coding for CD123 (A) and BDCA-2 (B) was evaluated by PCR in livers of controls (n = 3), NASH patients (n = 6), and HCV-infected, treatment-naive patients (n = 28). The * shows p < 0.05 compared with controls.

Our results suggested that circulating PDCs have increased baseline and TLR9-induced apoptosis in patients with chronic HCV infection. Reduced absolute numbers of the circulating PDCs were reported previously in patients with chronic HCV compared with controls (23–25). A HCV escape mechanism from IFN- 
-mediated antiviral effects would be most important in the liver, where HCV replicates and most of the virus and the virus-derived proteins are produced. High IFN- 
production has been described during the acute phase of HCV infection (32, 33); however, reports are contradictory with regards to chronic HCV infection (8–11). In the present study, we identified increased mRNA levels of the PDC markers, BDCA2 and CD123, suggesting that PDCs can be recruited to the liver in patients with chronic HCV infection. Consistent with this notion, CD80
, CD83
, and CD86
 cells with DC morphology were identified in livers of HCV-infected patients (34). The functional capacity of those PDCs in the liver remains to be evaluated. While our data shows PDC dysfunctions, reduced Ag-specific T cell activation and Ag presentation capacity of myeloid and monocyte-derived DCs may further contribute to the loss in virus-specific immune activation in chronic HCV infection (12, 35, 36).

Our observation that only patients with detectable levels of HCV, and not sustained responders or patients with NASH, encountered loss of PDCs and impaired IFN- 
production capacity suggested a direct role for the virus in the loss and functional impairment of PDCs. HCV encodes a long polypeptide that is cleaved into at least 10 known proteins (37). Core protein, the first to be cleaved from the viral polyprotein, has a distinct immuno-regulatory capacity. In agreement with previous studies (18, 38), we found that HCV core protein was found in the peripheral blood of all analyzed HCV-infected patients. Furthermore, we identified an inverse relationship between plasma core protein levels and circulating PDC frequency in patients with chronic HCV infection. Our studies revealed that HCV core protein induced IL-10 and TNF- 
production in monocytes, and these cytokines in turn resulted in PDC apoptosis and impaired capacity of PDCs to produce IFN- 
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that patients with HCV have a significantly higher prevalence of other blood-borne virus infections, including HIV, hepatitis B, CMV, as well as cryptococcus, tuberculosis, and sexually transmitted diseases. This suggests that patients with chronic HCV infection may indeed have signs of immunosuppression, leading to increased rate of other infections. Indeed, impaired IFN-α production and depletion of PDCs are common in viral infections, including HCV, human T cell leukemia virus type 1, and hepatitis B virus (50–54); however, the mechanisms of PDC depletion may not be identical in all viral infections. While direct cytolytic effect on PDCs is possible for retroviruses (HIV-1 and human T cell leukemia virus type 1), it is less probable for hepadnaviruses such as HBV and flaviviruses such as HCV. Distinct patterns of IFN-α production were described in patients infected with HIV-1 alone, HCV, or HIV-1/HCV coinfection (54), suggesting that different mechanisms account for the PDC functional impairments. Consistent with this, we found that unlike HCV core protein, HIV p24 or HBV core proteins did not inhibit IFN-α production in PBMCs (data not shown). We found that HCV used alternative tactics by acting on monocytes and affecting PDCs indirectly via monocyte-derived mediators. While our results offer a mechanistic explanation for HCV-induced PDC apoptosis via monocyte-mediated cytokine production, we cannot rule out the possibility that the increased baseline apoptosis found in PDCs of patients with chronic HCV infection would be due to a direct action of the virus on PDCs. Recent reports show the existence of extrahepatic sites of HCV replication, including PDCs (55). Interestingly, in vivo infection with HSV resulted in unresponsiveness of PDCs to subsequent in vitro rechallenge (56). Thus, we cannot rule out the possibility that HCV may directly affect PDCs in vivo, resulting in their apoptosis and unresponsiveness to subsequent TLR challenge in vitro, as found in our experiments.

In conclusion, we demonstrate that patients chronically infected with HCV have a profound defect in PDC frequency and IFN-α production capacity. Our results suggest that HCV patients’ PDCs are in vivo programmed for apoptosis and have diminished IFN-α-producing capacity. We identified that HCV core-induced IL-10 and TNF-α mediated these PDC defects, and removal of cytokine-producing monocytes normalized PDC IFN-α production even in the presence of HCV core protein. Our study indicates that viral-induced mechanisms of PDC loss and IFN-α production defects are likely to contribute to chronic viral persistence and may provide mechanistic explanations for the therapeutic benefits of IFN-α in HCV infection.

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Disclosures

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