Hepatitis C Virus Is a Weak Inducer of Interferon Alpha in Plasmacytoid Dendritic Cells in Comparison with Influenza and Human Herpesvirus Type-1

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Abstract
Plasmacytoid dendritic cells (pDCs) are responsible for the production of type I IFN during viral infection. Viral elimination by IFN-α-based therapy in more than 50% of patients chronically infected with hepatitis C virus (HCV) suggests a possible impairment of production of endogenous IFN-α by pDCs in infected individuals. In this study, we investigated the impact of HCV on pDC function. We show that exposure of pDCs to patient serum- and cell culture-derived HCV resulted in production of IFN-α by pDCs isolated from some donors, although this production was significantly lower than that induced by influenza and human herpesvirus type 1 (HHV-1). Using specific inhibitors we demonstrate that endocytosis and endosomal acidification were required for IFN-α production by pDCs in response to cell culture-derived HCV. HCV and noninfectious HCV-like particles inhibited pDC-associated production of IFN-α stimulated with Toll-like receptor 9 (TLR9) agonists (CpG-A or HHV-1) but not that of IFN-α stimulated with TLR7 agonists (resiquimod or influenza virus). The blockade of TLR9-mediated production of IFN-α, effective only when pDCs were exposed to virus prior to or shortly after CpG-A stimulation, was already detectable at the IFN-α transcription level 2 h after stimulation with CpG-A and correlated with down-regulation of the transcription factor IRF7 expression and of TLR9 expression. In conclusion, rapidly and early occurring particle–host cell protein interaction during particle internalization and endocytosis followed by blockade of TLR9 function could result in less efficient sensing of HCV by TLR9, with impaired production of IFN-α. This finding is important for our understanding of HCV-DC interaction and immunopathogenesis of HCV infection.

Introduction
Plasmacytoid dendritic cells (pDCs) are a highly specialized subset of dendritic cells that function as sentinels for viral infection and are responsible for production of large amounts of type I IFN during viral infection [1–3]. pDCs are able to detect genetic material of virus particles after their degradation in endosomal compartments via interaction with Toll-like receptors (TLR) [4]. pDCs are able to detect DNA of inactivated human herpesvirus types 1 (HHV-1) and 2 (HHV-2) via TLR9 (AAQ89443) [5,6], and they are able to detect single-stranded RNA of inactivated influenza virus and of HHV-1 via TLR7 (AAQ86869) [7–10]. However, inactivation renders some single-stranded RNA viruses, like measles [11], respiratory syncytial virus [11,12], and vesicular stomatitis virus [13], incapable of inducing potent pDC-associated production of IFN-α. The recognition of such viruses by TLR7 and the production of IFN-α (NP 076918) by pDCs require transport of cytosolic viral replication intermediates into lysosomes by the process of autophagy [13]. Recent results show that replicating HCV induces an autophagic response in immortalized human hepatocytes [14].

The eradication of hepatitis C virus (HCV) in more than 50% of chronically infected patients by treatment with IFN-α in combination with ribavirin [15,16] suggests that pDCs can play a major role in the control of HCV infection. Several studies that analyzed the function of pDCs in chronically infected patients compared with those from normal subjects reported a markedly reduced IFN-α production after ex vivo exposure of pDCs to agonists of TLR9 (e.g., CpG oligonucleotides) and TLR7 (imidazoquinoline components, e.g., R848, resiquimod) [17–20]. However, other reports found no difference between these groups [21,22]. Whereas in these studies, the pDCs obtained from patients with chronic HCV infection were exposed to synthetic stimulators of TLR7 or TLR9 in the absence of HCV, more recent studies investigated the effects of TLR7 or TLR9 ligands on pDCs purified from healthy donors in the presence of cell culture-prepared HCV (HCVcc) [21,23]. These reports have shown that exposure of pDCs from healthy donors to HCVcc is not followed by expression of the HCVcc genome and viral replication, that HCVcc does not induce pDC-associated production of IFN-α and cell differentiation [21,23], and that, in addition, HCVcc blocks IFN-α production mediated via TLR9 [23].
In contrast to these earlier studies, we show here that exposure of pDCs to HCV results in production of IFN-α by pDCs isolated from some donors, although this production is significantly lower than that induced by influenza and human herpesvirus type 1 (HHV-1). Production of IFN-α was sensitive to specific inhibitors of endocytosis and endosomal acidification and was resistant to virus inactivation. In order to better understand the mechanism of poor induction of IFN-α by HCVcc-exposed pDCs, we also studied the inhibition of TLR9-mediated IFN-α production with HCVcc [23] and with HCV-like particles (HCV-LPs) [24–26]. We conclude that the interaction of the viral particle with host cell factors during viral internalization and endocytosis followed by blockage of TLR9 signaling could result in less efficient sensing of HCV RNA by TLR7, with impaired production of IFN-α. On the basis of these results we propose a new mechanism by which HCV can evade recognition by pDCs.

Results

HCV does not induce maturation of purified pDCs

First, we compared the capacity of molecular clone HCVcc JFH-1 to induce pDC differentiation with the capacities of resiquimod, influenza virus A/H3N2/Johannesburg, and HHV-1 KOS (Figure 1). Since TLR7 recognizes and is activated by viral RNA [27,28], and TLR9 by viral DNA [5,29], we normalized the quantity of assayed viruses on the basis of the number of virus genome copies as determined with PCR (not shown). Purified pDCs from normal healthy donors (Figure 1A) were inoculated with examined viruses at a multiplicity of 100 genome copies per cell. All viruses were purified by ultracentrifugation through a cushion of 20% sucrose to minimize the presence of bystander activation factors. Analysis of the purity of concentrated stocks of HCVcc by electron microscopy showed the presence of particles of 60–80 nm in diameter and the absence of detectable amounts of cellular DNA and RNA (supplementary Figure S1).

One-day-culture of pDCs in medium supplemented with IL-3, an important factor for pDC survival, resulted in partial pDC maturation (Figure 1A). pDC viability declined with a half-time of approximately 1 day (Figure 1B). Flow cytometry analysis of Annexin V/7ADD-stained cells revealed no increase in the proportion of apoptotic cells among pDCs inoculated with HCVcc in comparison with non-stimulated or resiquimod-stimulated pDCs (Figure 1C–E). Inoculation of pDCs from 10 healthy donors with HCVcc slightly but significantly down-regulated the expression of CD80 (ABK41933) and CD86 (CAG46642), whereas the expression of other assayed surface markers (CD40 (AAL92994), CD83 (CAB63843), and CCR7 (NP 001829)) remained unchanged in comparison with culture without stimulation (Figure 1F and 1G).

In contrast to influenza virus and HHV-1, HCV is a weak inducer of pDC-associated production of IFN-α and TNF-α

Next, we compared the capacity of HCVcc JFH-1 to induce pDC-associated secretion of IFN-α and TNF-α (CAI41940) with the capacities of HCV-1 LAI, influenza virus A/H3N2/Johannesburg, and HHV-1 KOS. pDC-associated production of IFN-α increased with increasing levels of HCV genome copies, although at a lower rate as observed for HCV JFH-1 CAI41940. Taken together, these data suggest that exposure of pDCs to cell culture- or patient-derived HCV resulted in production of IFN-α, although it showed a high degree of variability among pDCs from different donors.

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Complementing the investigation of IFN-α production, we studied the stimulatory effect of the four viruses on pDC-associated TNF-α production (Figure 3C). Similar as seen for IFN-α,
incubation of pDCs with HCV JFH-1 resulted in a dose-dependent production of TNF-α, albeit the level of TNF-α was markedly lower than the level of TNF-α induced by influenza virus and HHV-1. Thus, we conclude that HCV, compared to influenza virus and HHV-1, is a weak inducer of DC-associated production of IFN-α and TNF-α.

Activation of pDCs by HCV occurs during viral endocytosis

To investigate whether endocytosis was required for recognition of HCV, we tested several inhibitors that block the cellular uptake of particular structures, including viruses (Table 1). Dimethyl amiloride, cytochalasin D, and chlorpromazine fully inhibited IFN-α secretion, suggesting that viral endocytosis is required for pDCs stimulation. Incubation of pDCs with chloroquine, quinacrine, and bafilomycin A1 resulted in complete inhibition of HCV-cc-induced IFN-α secretion, showing that acidification/maturation of the endosomes is necessary for activation of pDCs. Although less effective than chloroquine, quinacrine or BafA1, NH₄Cl inhibited HCV-cc-induced IFN-α secretion in a dose-dependent manner. Consistent with previous observations that activation of TLR7 occurs within endosomes, inhibition of IFN-α production by inhibitors of endocytosis was also observed in response to resiquimod [31]. In conclusion, our findings suggest that endocytosis of the HCV particle, with subsequent acidification of endosomes, is required for pDC activation.

Inactivated HCV stimulates similar levels of pDC-associated production of IFN-α as replication-competent infectious virus

The majority of viruses that are presented to TLR by endocytosis has a similar IFN-α stimulatory potential whether they are exposed to pDCs in replication-competent or inactivated form [5-7,9]. Exposure of pDCs to inactivated virus lead to reduction of IFN-α secretion by only 8% (thermo-inactivated virus) or 18% (UV-inactivated virus), whereas the infectious titers reduction of IFN-α of HCV-cc injected in Huh7.5.1 producer cells (TCID₅₀Huh7.5.1/ml) dropped 10,000-fold or 1,000-fold, respectively (Table 2). Similar levels of pDC-associated production of IFN-α stimulated with inactivated virus with replication-competent infectious virus indicate that, HCV does not need to be replication-competent to induce IFN-α production in infected cells and is compatible with a major role of endocytosis in pDC activation.

Exposure of pDCs to HCV-cc prior to or shortly after CpG-A stimulation blocks TLR9-mediated production of IFN-α

To investigate further the impact of HCV on pDC function, we studied kinetics of disruption of TLR9-mediated production of IFN-α. For this reason, we exposed the cells before or after stimulation with CpG-A to HCV-cc, as shown in the chart flow protocol (Figure 4A). Priming with HCV-cc 2 h before CpG-A stimulation (Figure 4A, line a) reduced the CpG-A-induced IFN-α levels by >90% (Figure 4B). Less pronounced (69%) inhibition of TLR9-mediated activation by HCV-cc was observed when pDCs were exposed to HCV-cc and CpG-A concomitantly (Figure 4A, line b and 4B). The inhibitory effect of HCV-cc almost disappeared (12% inhibition) when the virus was added 1 h after CpG-A (Figure 4A, line e, and 4B). Thus, HCV-cc inhibits TLR9-mediated production of IFN-α only when inoculation with HCV-cc precedes or follows shortly after CpG-A stimulation.

To study events that precede secretion of IFN-α, we examined the expression of IFN-α, IRF7, TLR7, and TLR9 genes in pDCs...
that had been stimulated with Cpg-A, with HCV JFH-1, or with both HCV JFH-1 and Cpg-A (Figure 4A, line a, and 4C). In four experiments with pDCs purified from three donors, Cpg up-regulated IFN-α expression in pDCs from 2,100- to 12,000-fold by 4 h, while simultaneous stimulation with HCVcc reduced the up-regulation of IFN-α expression by 18 to 76 times. Relative IFN-α expression then dropped down approximately 10 times after 16 h of Cpg stimulation, in comparison with the level of expression after 4 h. In pDCs purified from different donors HCV up-regulated IFN-α expression from 1 to 44 times.

Since the ability of Cpg-A to induce IFN-α was severely impaired in the presence of HCVcc, we examined TLR7 and TLR9 expression in Cpg-A-stimulated cells. Incubation of pDCs with Cpg-A resulted in up-regulation of TLR7 expression in pDCs after 2 h and later. By contrast, expression of TLR9 was down-regulated. Priming of pDCs with HCVcc slowed down the TLR7 up- and TLR9 down-regulation by approximately 50%. Because triggering of TLR9 and/or TLR7 is known to activate the IRF-7-mediated pathway, we also examined the expression of this molecule, which is responsible for the transcription of IFN-α, and which is constitutively present in pDCs (Figure 4C). Modest up-regulation (1.6 times) of IRF-7 expression in Cpg-A-stimulated pDCs was reduced by approximately 50% in HCVcc-prime cells. Taken together, blockade of stimulation via TLR9 occurs early after exposure of pDCs to HCV and correlates with down-regulation of IRF7.

HCVcc and HCV-LPs but not HCV core or envelope glycoprotein E2 inhibit pDC-associated production of IFN-α stimulated via TLR9

We assayed whether non-infectious HCV-LPs, HCV core (BAA01000), and envelope glycoprotein E2 disrupt TLR9-mediated production of IFN-α, in addition to the reported effect of HCVcc [23]. pDCs primed with HCVcc for 2 h and subsequently stimulated with Cpg-A produced IFN-α at 11.2±5.4% of the levels produced by pDCs stimulated only with Cpg-A (Figure 5A). This reduction was highly significant relative to that in Cpg-A-treated cells from 10 healthy donors in 14 experiments (p = 0.0002) and relative to control supernatants from Huh7.5 cells transfected with subgenomic replicon and rendered apoptotic by UV irradiation (p = 0.01). Inhibition of TLR9-mediated production of IFN-α was HCVcc dose-dependent and dropped to negligible level in pDCs exposed in average to one HCVcc RNA molecule per cell.

To test the biological relevance of results obtained with cell culture-derived HCV, we determined the level of inhibition of TLR9-mediated activation by patient serum-derived HCV (Figure 5A). pDCs primed for 2 h with HCV from patients (n = 5) and subsequently stimulated with Cpg-A produced IFN-α at 19.3±5.9% of the levels produced by pDCs stimulated only with Cpg-A, whereas pDCs primed with sediments prepared by ultracentrifugation through a cushion of 20% sucrose of sera obtained from four HCV-negative individuals produced IFN-α at 58.3±11.6% of the levels produced by pDCs stimulated only with Cpg-A (Figure 5A). The difference between inhibition of TLR9-mediated activation by HCV from patients’ serum and healthy controls was significant (p = 0.032).

In addition to Cpg-A, we tested the effect of HCV on the stimulation of pDCs by HHV-1, a naturally occurring TLR9 agonist (Figure 5A). Exposure of pDCs to HCV followed by inoculation with HHV-1 reduced IFN-α secretion by >60% in comparison with pDC culture exposed only to HHV-1. Control HCV virion-free supernatant from Huh7.5 cells transfected with HCV subgenomic replicon did not significantly block HHV-1-induced IFN-α secretion.

To determine whether replication-competent HCV is required for the inhibitory effect, pDCs were exposed to heat-inactivated or UV-inactivated virus or to HCV-LPs in the presence of Cpg-A. Heat-inactivated as well as UV-inactivated HCVcc suppressed Cpg-A-induced IFN-α secretion by >80%, as did the replication-competent virus (Figure 5A).

| Table 1. Sensitivity of IFN-α production to inhibitors of endocytosis. |
|---------------------------------------------------------------|
| **Endocytosis inhibitor** | IFN-α production, %* | **HCVcc** | **0.5 µM resiquimod** |
|-------------------------------|-----------------|---------|-----------------|
| Dimethyl amiloride (50 µM)    | <5%             | <5%    | <5%             |
| Cytochalasin D (10 µM)        | <5%             | <5%    | <5%             |
| Chlorpromazine (6.25 µM)      | <5%             | <5%    | <5%             |
| Chloroquine (5 µM)            | <5%             | <5%    | <5%             |
| Quinacrine (5 µM)             | <5%             | <5%    | <5%             |
| NH4Cl (1 mM)                  | 80%             | 45%    | 45%             |
| NH4Cl (10 mM)                 | <5%             | <5%    | <5%             |
| Bafilomycin A1 (50 nM)        | <5%             | <5%    | <5%             |

*Percentage of mock-treated control. pDCs (300,000 cells) were exposed to 3×106 HCVcc RNA copies in a final volume 300 µl for 16 h.

doi:10.1371/journal.pone.0004319.t001

| Table 2. Exposure of pDCs to heat-inactivated and UV-inactivated virus stimulates production of IFN-α.|
|---------------------------------------------------------------|
| **TCID50-huh7.5/ml** | **TCID50-huh7.5/ml** | **IFN-α** | **IFN-α** |
|---------------------|---------------------|-----------|-----------|
| HCV                 | 105                | 100       | 120±38    | 100       |
| HCV, 30 min 56 °C   | <101               | <0.01     | 111±25    | 92        |
| HCV                 | 105                | 100       | 175±42    | 100       |
| HCV, UV (0.2 J/cm²) | 0.1                | 142±40    | 81        |

*106 HCVcc RNA copies and 105 pDCs per milliliter.

doi:10.1371/journal.pone.0004319.t002
To further define the viral factors involved in this mechanism, we primed pDCs with non infectious HCV-LPs instead of HCV (Figure 5A). HCV-LPs are generated by self assembly of the HCV structural proteins in insect cells and have been shown to have similar biophysical, antigenic and immunogenic properties as HCV particles [25,32–34]. Due to the lack of a functional genome and the nonstructural proteins, HCV-LPs are noninfectious. Since DCs efficiently take up, process and present HCV-LPs to HCV-specific CD4+ and CD8+ T cells, the interaction of HCV-LPs with DCs has allowed to identify mechanisms of HCV uptake and cross-presentation by human dendritic cells [25,26]. Priming with HCV-LPs reduced the CpG-A–induced IFN-α levels by >75% compared to priming with control preparations. The absent inhibition of CpG-A–induced IFN-α production by control cell preparations produced from insect cells infected with a control baculovirus ruled out that contaminating cellular proteins of virus preparations were responsible for blocking TLR9 function. In contrast to HCVcc and HCV-LPs, HCV E2 (AAB30986) or core protein did not inhibit TLR9-mediated activation of pDCs. In conclusion, these data indicate that HCV virions or virus-like particles, but not the soluble forms of core and envelope glycoprotein E2 suppress stimulation via TLR9.

Figure 4. Kinetics of disruption of CpG-A-mediated stimulation of pDCs with HCVcc. (A) Flow chart protocol showing inoculation with HCV (solid arrows), treatment with CpG-A (dashed arrows), and IFN-α assay determined by ELISA. (B) Percentage of IFN-α secreted by pDCs that were stimulated with CpG-A and HCV relative to IFN-α secretion by pDCs stimulated only with CpG-A. Purified pDCs were inoculated with 100 HCVcc RNA copies per cell. (C) Effect of HCVcc on the expression of IFN-α, IRF-7, TLR7 and TLR9 mRNA. pDCs were primed with HCVcc and stimulated with CpG-A as shown in panel A, line a. The gene expression levels were determined with real-time PCR were normalized to GAPDH expression. Data are presented as fold induction over medium control at time zero (given the value of 1.0) and are from one of three representative experiments. doi:10.1371/journal.pone.0004319.g004

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In addition to production of large amounts of IFN-α, TLR activation of pDCs can induce the production of proinflammatory cytokines such as TNF-α and IL-6. Similar to the marked inhibition of TLR9-mediated induction of IFN-α, exposure of pDCs with cell culture-derived HCV resulted in a more than 80% inhibition of CpG-A-induced TNF-α production (Figure 5B). A less pronounced effect of HCV on TNF-α production (blockade >40%) was observed when the cells were stimulated by HHV-1, a naturally occurring TLR9 agonist, instead of CpG-A (Figure 5B).

In marked contrast, HCV exerted no discernible effect on pDC response to TLR7-mediated induction of IFN-α secretion (Figure 5C). Neither HCV (12 experiments with pDCs from nine healthy donors), nor heat-inactivated HCV, nor HCV E2, nor patient-derived HCV, nor HCV-LPs, nor cell-free supernatant from Huh7.5 cells transfected with subgenomic replicon suppressed pDC-associated secretion of IFN-α induced with a synthetic ligand (resiquimod) or with a natural agonist (influenza virus) (Figure 5C). Thus, the HCV-induced suppression of TLR9-mediated IFN-α secretion is not due to a generalized effect of HCV on TLR signaling in pDCs.

**Discussion**

In the present study, we demonstrate that HCV, in contrast to influenza virus or HHV-1, is a weak inducer of IFN-α in purified pDCs from healthy donors, and it does not induce pDC maturation. As in our study, no maturation was detected in pDCs from HCV-infected individuals [21,23]. Sensitivity of IFN-α production to inhibitors of endocytosis (dimethyl amiloride, cytochalasin D, and chlorpromazine) suggests that HCV virions or virus-like particles, but not the soluble forms of core and envelope glycoprotein E2 suppress stimulation via TLR9.
primed with HCV virus particles (100 HCV genomes per cell) prepared the same way as the viral stock (Sg-replicon, UV). pDCs were also primed (HCV 1 KOS (multiplicity of infection = 100), or (C) with TLR7-agonists, with approximately 5,000 particles per cell) or to control (ctrl) cell envelope glycoprotein E2 (10^5 g/ml). pDC were also incubated with HCV core or transfected with HCV subgenomic replicon at 30 mJ/cm² prepared in control supernatant collected 16 h after UV irradiation of Huh7.5 cells inactivated with heat-treatment at 56°C for 30 min (HCVcc, 56°C) or alternatively, pDCs were primed with the same quantity of virus with UV-treatment at 0.2 J/cm² (HCVcc, UV), or exposed to noninfected serum (M). Secretion of IFN-α in pDC-cultures; pM-W, Mann-Whitney two-tailed non-parametric test used to compare differences between the distributions of IFN-α production in primed and non-primed pDC-cultures; pM-W, Mann-Whitney two-tailed non-parametric test used to compare differences between the distributions of IFN-α production in HCV-exposed and Sg-replicon-exposed pDC cultures, or in HCV-serum-exposed and HCV-serum-exposed pDC cultures. doi:10.1371/journal.pone.0004319.g005

**Figure 5.** Effect of HCV, HCV-LPs, HCV core, and envelope glycoprotein E2 on the secretion of IFN-α from pDCs primed with TLR9 or TLR9 agonists. (A) Purified pDCs were exposed to 100, 10, or 1 HCVcc RNA copies per cell or to patient sera-derived HCV. Alternatively, pDCs were primed with the same quantity of virus inactivated with heat-treatment at 56°C for 30 min (HCVcc, 56°C) or with UV-treatment at 0.2 J/cm² (HCVcc, UV), or exposed to noninfectious insect cell-derived HCV-LPs (0.1 μg E2/ml corresponding to approximately 5,000 particles per cell) or to control (ctrl) cell preparations (1 μg/ml). pDC were also incubated with HCV core or envelope glycoprotein E2 (10 μg/ml), or with an equivalent volume of control supernatant collected 16 h after UV irradiation of Hu7.5 cells transfected with HCV subgenomic replicon at 30 mJ/cm² prepared in the same way as the viral stock (Sg-replicon, UV). pDCs were also primed with HCV virus particles (100 HCV genomes per cell) prepared from five different sera of chronically infected patients (HCV⁺ serum) or from equivalent volumes of four different sera of healthy individuals (HCV⁻ serum). Two hours later, primed or mock-primed pDCs were stimulated (A) with TLR9-agonists, with CpG-A (2.5 μg/ml), or with HHV-1 KOS (multiplicity of infection = 100), or (C) with TLR7-agonists, with resiquimod (R848, 0.5-μM), or with influenza virus A/H3N2/Johannesburg (multiplicity of infection = 100). Secretion of IFN-α (A, C) or TNF-α (B) in cell-free supernatant of pDCs was determined by means of ELSA analysis 1 day post-stimulation. The results are expressed as percentages of IFN-α production from pDCs that were first primed as specified above and then further treated with the respective TLR agonist relative to IFN-α production from pDCs stimulated only with the respective TLR agonist. pM-W, Wilcoxon matched pairs test used to compare differences between the distributions of IFN-α production in primed and non-primed pDC-cultures; pM-W, Mann-Whitney two-tailed non-parametric test used to compare differences between the distributions of IFN-α production in HCV-exposed and Sg-replicon-exposed pDC cultures, or in HCV-serum-exposed and HCV-serum-exposed pDC cultures. Differences in the number of assayed donors, in the viral strain, and virus titer could be the reason why no production of IFN-α was detected in in vitro HCV-exposed in some other studies [21, 23]. Limited production of IFN-α as well as the absence of pDC differentiation may contribute to the reduced innate and adaptive immune responses against HCV observed in the course of chronic infection [41–45]. Both HCV and HIV-1, which are related to chronic diseases accompanied by sustained plasma viremia, are weak inducers of IFN-α and TNF-α. In contrast, influenza virus and HHV-1, the potent inducers of IFN-α and TNF-α are related to diseases with transient viremia.

In order to better understand the low production of IFN-α by HCV-exposed pDCs, we studied HCV-induced blockage of TLR9-mediated production of IFN-α. We demonstrate that in addition to HCVcc [23], HCV-LPs also block TLR9-mediated production of IFN-α. It has recently been demonstrated that pDCs cultured in the presence of IL3 bind HCV-LPs [25]. Blockage of CpG-A-induced production of IFN-α by HCV-LPs unequivocally shows that replication-competent phenotype and expression of the nonstructural proteins of HCV are not required for the inhibition. Furthermore, these data demonstrate that an interaction of the viral particle with host cell factors during viral uptake and endocytosis is involved in inhibitory mechanism. Since recombinant soluble core and E2 proteins could be found in different compartments to HCV nonstructural proteins of HCV are not required for the inhibition. In contrast, influenza virus and HHV-1, the potent inducers of IFN-α and TNF-α are related to diseases with transient viremia.
released from hepatocytes probably occurs. Further studies are underway to fine map the factors within the viral particle and to identify the host cell proteins mediating this effect.

Several mechanisms could be responsible for the poor responsiveness of pDCs to HCV on one hand, and the blockage of TLR9-mediated production of IFN-α on the other. It is conceivable that the poor secretion of IFN-α by pDCs could be related to HCV cross-linkage of a variety of cell surface receptors that down-regulate IFN-α production, such as BDCA-2 (Q8WTT0) [47,48], DCIR (NP 919429) [49], ILT7-FcεRI gamma (AAD02039) [50], and FcεRI (CAAA6953) [51]. Among them, ligation of DCIR is followed by co-localization of DCIR and CpG-A in endosomes, which results in a specific TLR9- but not TLR7-mediated inhibition of IFN-α and TNF-α [49]. Inhibition of both IFN-α and TNF-α was seen also in our experiments with HCVcc (Fig. 3A,B). It is conceivable that HCVcc and HCV-LPs are, after ligation of a cell surface receptor, transported to the vicinity of TLR9 and that they use (as a “wrong cargo”) the mechanism of spatiotemporal regulation of IFN-α induction [52] to escape from recognition by TLR7, the presumed natural receptor for HCV [53].

The blockade of TLR9-mediated production of IFN-α, effective only when pDCs were exposed to virus prior to or shortly after CpG-A stimulation, was already detectable at the IFN-α transcription level 2 h after stimulation with CpG-A and correlated with down-regulation of IRF7 expression. Whereas TLR7 and TLR9 signal transduction pathways downstream of Toll-IL-1R overlap, the regulation of the gene expression of TLR7 and that of TLR9 are substantially different. Up-regulation of TLR7 expression and down-regulation of TLR9 expression in CpG-A-stimulated pDCs, observed also in previous studies [54,55], had been reduced to approximately 50% after pre-stimulation with HCV JFH-1. Constitutively lower expression levels of TLR9 compared to TLR7 in normal pDCs [56,57] could enhance the former mechanisms and make TLR9 more vulnerable to inhibitory effects. Because pDCs stimulated through TLR9 are refractive to re-stimulation [58], we suppose that host cell proteins potentially involved in both traffic of the viral particle and inhibition of TLR signaling, make pDCs non-responsive to the second signal given by CpG-A. Taken together, rapidly and early occurring HCV particle-host cell protein interaction during particle internalization and endocytosis is followed by blockade of TLR9 by cellular host protein with impaired production of IFN-α. Supposed sequestration of viral particle in the proximity of TLR9 could result in less efficient sensing of HCV RNA by distal TLR7, without affecting TLR7 function, as shown by responses to resiquimod. Triggering the endocytosis of host molecule(s) that inhibit TLR9 signaling and transport virus particles toward TLR9, out of contact with TLR7, could represent a new mechanism by which HCV evades the immune system.

In spite of our efforts to minimize the presence of bystander activation factors in viral stocks, such as preparations of HCV JFH-1, it is possible that the virus preparations were contaminated with membranous vesicles and other RNA- and DNA-containing cellular components that were co-purified with the virus. This cellular material could theoretically participate, in addition to HCV, in the stimulation of pDC-associated production of IFN-α and in the suppression of CpG-A-induced IFN-α secretion from pDCs exposed to HCV. To address this issue, we stimulated pDCs with a suspension prepared from cell-free supernatant of apoptotic Huh7.5 cells transfected with HCV subgenomic replicon. This control HCV-free supernatant did not induce pDC-associated production of IFN-α and did not block CpG-A-induced IFN-α secretion. Furthermore, similar levels of IFN-α secreted from pDCs stimulated by HCV virions purified from different biological materials—recombinant cell culture-derived as well as patient-derived HCV—further confirm the induction of pDC-associated IFN-α by HCV and not by cellular components. Side-by-side control experiments using preparations of cell lysates containing all proteins or cellular factors potentially contaminating particle preparations confirm that HCV-LPs, and not contaminating material, blocked CpG-mediated activation of pDCs.

Variability of the levels of IFN-α produced by the HCV-exposed pDCs from different donors could reflect polymorphism of the HCV-induced inhibitory mechanisms and could result in different outcomes of HCV infection (spontaneous resolution versus chronicity). Optimal viral concentration that blocked CpG-A-mediated production of IFN-α by isolated pDCs corresponded to 10^6 HCVcc RNA molecules (and approximately to 5 x 10^5 HCV-LP particles) per milliliter. Only marginal inhibition was observed at 100 times lower HCVcc RNA concentration. Given that most chronically infected patients have levels of HCV RNA between 10^4 and 10^6 copies per milliliter, the virus concentration necessary for in vitro inhibition of TLR9-mediated production of IFN-α is compatible with the block of IFN-α in approximately 0.1 to 10% of circulating pDCs, and with the observation that individuals chronically infected with HCV are not immunocompromised.

Similar to our results obtained for HCV, several other viruses have been shown to block stimulation via TLR9. As recently shown by Fauci and colleagues [59], HIV-1 gpl20 (AAC37925) - a BDCa-2 [59] and DCIR ligand [60] - inhibits TLR9-mediated activation and IFN-α secretion, but not TLR7-mediated activation and IFN-α secretion in pDCs. Hepatitis B virions also selectively inhibit TLR9-mediated activation and IFN-α secretion (I. E. Vincent, C. Trepo, personal communication [61]), showing that two hepatitis viruses impair the same pDC function.

Obvious caveats must be considered in transposition of in vitro results based on analysis of isolated pDCs exposed to HCVcc in an “acute setting” [23], to ex vivo experiments based on analysis of complex interactions of pDCs with monocytes/macrophages [19,62] and NK cells [63] in “chronical setting”, and to pathogenesis of HCV in infected individuals. A better understanding of the stimulation of TLR7 and TLR9 with their synthetic ligands in the presence of HCV may identify new approaches for the development of antiviral strategies based on TLR agonists. Recent clinical studies have shown that administration of TLR7 agonists resiquimod [64] and isotaribine [65], as well as of TLR9 agonist CpG-A [66] results in reduction of plasma virus concentration in patients with chronic HCV infection.

**Materials and Methods**

**pDC isolation and culture**

We prepared peripheral blood mononuclear cells (PBMCs) using density gradient centrifugation on Lymphoprep (AbCys S.A., Paris, France). pDCs were purified directly from PBMCs by use of magnetic bead isolation kits followed by separation on AutoMacs (Miltenyi Biotech). The BDCA-4 diamond isolation kit (Miltenyi Biotech) was used and yielded levels of purity from 75% to 95%, with a contamination of less than 5% myeloid dendritic cells. In some experiments in which we wanted to achieve the highest purity of pDCs, we pre-enriched dendritic cells from PBMCs to 50–70% purity by means of magnetic bead depletion of CD14+ monocytes/macrophages, CD11b- B- and NK-cells using MAb against CD3-CD19-CD56-CD14-CD34-CD16-CD66b and glycoporin A (Human Dendritic Enrichment Kit: Stem Cell Technologies, Inc., Grenoble, France). We then separated pDCs from the enriched population by means of fluorescence-activated cell sorting (FACS-ARIA, Becton-Dickinson Bioscience, Erembo-
Production and purification of HCV-LPs

HCV-LPs derived from the cDNA of the infectious clone H77 were expressed and purified as described previously [24] [26]. Control preparations were derived from insect cells infected with a recombinant baculovirus containing the cDNA for β-glucuronidase (GUS) [24,26]. The quantity of HCV-LPs was determined by analysis of the HCV-LP E2 concentration using an E2-specific ELISA [69] [26]. An HCV-LP E2 concentration of 0.1 μg/ml corresponded to approximately 5 × 10^8 virus particles/100 μl or about 5,000 viral particles per cell (estimation of particles according to [32]).

Production and purification of cell culture-derived HCV (JFH1)

The HCV genotype 2A clone JFH-1, derived from a Japanese patient with fulminant hepatitis [67,68], was prepared as previously described in detail [67]. Briefly, plasmid pHJFH1 (kindly provided by T. Wakita, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) was used as a template for in vitro transcription with the MEGAscript™ T7 kit (Ambion, Austin, TX, USA). We then electrophoretically HCV RNA (10 μg) in 5 × 10^7 Huh7.5.1 cells (kindly provided by S. L. Wieland and F. V. Chisari, The Scripps Research Institute, La Jolla, CA) at 270 V and 960 μl using a Bio-Rad Gene Pulser system. Transfected cells were then transferred to complete DMEM supplemented with 10% fetal calf serum and passaged every 3–5 days. The infectious endpoint titers of HCV-cc were determined from production of cytopathic effect after infection of Huh7.5.1 cells with tenfold dilutions of virus-containing cell-free supernatant in duplicate. We prepared virus stocks by infecting 10^7 Huh7.5.1 cells with 10^7 TCID_Huh7.5.1 of JFH-1 virus harvested from an RNA transfection experiment. Maximum titers of up to 10^6 TCID_Huh7.5.1/ml in the supernatant were reached between 14 and 20 days post-infection. The titers of HCV-cc genome-containing virus particles determined routinely with semiquantitative RT-PCR were 100 to 1,000 times higher than infectious titers. After 1,000-fold concentration by ultracentrifugation, the infectious titers typically increased 20-fold, whereas the titers of HCV genome-containing virus particles increased approximately 200-fold.

As a control, we used cell-free supernatant from Huh7.5.1 cells transfected with H/SG-neo (L+) subgenomic replicon [30] (kindly provided by C. M. Rice, The Rockefeller University, New York, NY) UV-irradiated at 30 mJ/cm^2 in a UV Stratalinker 1800 equipped with an integral UV photometer (Stratagene, La Jolla, CA) as a control. HCV viral particles from the serum samples of infected patients and supernatants from Huh7.5.1 cells transfected with H/SG-neo (L+) subgenomic replicon were ultracentrifuged and purified under the same conditions.

HCVcc virus inactivation

Concentrated virus at 10^8 RNA copies/ml was inactivated by heat treatment for 30 min at 56°C or by exposure to 0.2 J/cm^2 UV with a UV Stratalinker 1800 equipped with an integral UV photometer (Stratagene, La Jolla, CA). pDCs were inoculated with virus in a quantity equivalent to 100 genome-containing virus particles per cell. This quantity of genome equivalents corresponded to an HCV infectious dose of 0.01–0.1 tissue culture infectious doses per Huh7.5.1 indicator cell.

Quantitation of viral genome copies

Using the QIAamp viral RNA kit (Qiagen, Hilden, Germany), we isolated RNA from virions present in the ultracentrifuged virus. We determined endpoint dilution titers of viral genome copies with semiquantitative RT-PCR using the Superscript One Step RT-PCR system (Invitrogen, Cergy Pontoise, France). The HCV 5' untranslated region was amplified by means of nested PCR as described previously [71]. Alternatively, HCV RNA was quantified on the basis of a real-time reverse transcription polymerase chain reaction using the primer RTU1 [71] for cDNA synthesis, primer pair UTR2 and RTU2 [71] for PCR amplification, and HCV/JFH-1 RNA prepared in vivo by means of T7 polymerase as a standard.

For PCR amplification of the HIV LTR, we used the following primer pairs: 5'-CTGTGAGATCTCAGACACACAAAGGCTAC (sense; L2) and 5'-GCTGCTATTATGTAGCATCTGAGGCC (antisense).
were tested after 20 h for the IFN-β with bafilomycin A1 (50 nM), all from Sigma-Aldrich. Supernatants at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Gene expression assay and analysis
We extracted total RNA using the Qiagen RNeasy micro kit (Qiagen, Courtaboeuf, France) and converted it to cDNA using random hexamers (High Capacity cDNA RT kit, Applied Biosystems, Courtaboeuf, France). We assayed human IFN-α, IFN-β, TLR7, and TLR9 using Taqman Gene Expression Assay primers with labeled probes (Applied BioSystems). Threshold cycle (Ct) values for each gene were normalized to GAPDH. The negative control for each experiment, stimulation of pDCs with medium alone, was assigned a value of 1, and all data are expressed as fold induction over the negative control.

Immunofluorescence analysis
For analysis of cell surface marker expression, cells were incubated for 15 min at room temperature in the presence of FITC-conjugated CD80, FITC-conjugated CD83, PE-conjugated CD40, PE-conjugated CD86, PE-Cy7-conjugated CD123, and PE-Cy7-conjugated HLA-DR MAbS (all purchased from BD or Beckmann Coulter). pDC apoptosis was detected using FITC-conjugated Annexin V and 7-amino-actinomycin D (7ADD) in Annexin V-binding buffer (Becton-Dickinson). pDCs were gated based on side and granularity (FSC/SSC) and analyzed for the presence of fluorescent cells. After labeling, cells were fixed in 4% paraformaldehyde and analyzed after gating on live CD123+/HLA-DR+ cells with a FACS-ARIA using DIVA software (Becton-Dickinson, Le Pont de Claix, France). Data were analyzed by means of Flowjo software.

Determination of IFN-α and TNF-α production by ELISA
To measure the quantities of total IFN-α and TNF-α produced, we collected supernatants from parallel cultures after 16 to 20 h and assayed them using a human IFN-α ELISA (PBL Laboratories) or a TNF-α ELISA (Opti-EIA set, Pharmingen), respectively.

Statistical analysis
To compare the levels of IFN-α production by pDCs exposed to different viruses, we used a nonparametric Mann-Whitney test. To compare responses to TLR agonists in the presence versus absence of HCV, we used the Wilcoxon matched pairs test. Data were analyzed with Prism 4 Biostatistics software. All tests of significance were two-sided, and a p value ≤0.05 was considered to be significant.

Supporting Information
Figure S1 Purity of viral preparations. (A) Concentrated supernatant from Huh7.5.1 cells infected with HCV JFH-1 or similarly concentrated supernatant from minireplicon transfected Huh7.5.1 cells collected 16 h after UV irradiation with a 30 mJ/cm2 dose were adsorbed onto collagen membrane-coated electron microscopy grids. The adsorbed materials were negatively stained with 1% uranyl acetate or with 1% sodium phosphotungstate and observed in a Zeiss MET-EM 912 microscope. The presence of virus-like particles of 60–80 nm in diameter in the negatively stained viral preparation is shown by arrows. (B) Viral like particles or exosomes are absent in supernatant from Huh7.5 cells transfected with subgenomic (Sg) replicons. (C) We determined the quantity and quality of RNA present in the viral preparation using the Agilent 2100 bioanalyzer and RNA LabChip® kit. Contamination of virus preparation with RNA material was below the detection limits of the control methods used in our experiments (≤1 ng of RNA/ml). To determine the level of contamination of viral preparations with cellular DNA, we also amplified by means of GAPDH-specific PCR the DNA molecules presumably present in 5-μl aliquots (5×106 genome-containing virus particles) of viral stock used to stimulate pDC cultures. No GAPDH-specific signal was detected in 4 assayed aliquots (not shown).

Figure S2 Secretion of IFN-α induced with molecular clone HCV JFH-1 and with resiquimod in pDCs from different normal healthy donors. Cell cultures of pDCs purified from different normal healthy donors, adjusted to a concentration of 106 cells/ml in the presence of IL-3, were inoculated with 100 HCV RNA-containing virus particles per cell or stimulated with resiquimod (884, 0.5 μM) in a total volume of 200 μl. Secretion of IFN-α in cell-free supernatant was determined by means of ELISA analysis 1 day post-stimulation. Each point represents a different donor analyzed in Figure 1.

Acknowledgments
We thank C. M. Rice, R. Longman, and L. Dustin for critical reading of this manuscript, T. Wakita for providing the HCV JFH1 clone, C. M. Rice for the H/SG-neo (L+) subgenomic replicon, and S. L. Wieland and F. V. Chisari for Huh7.5.1 cells. We are indebted to M. Melioli and A. Leon (Vivalis, SA) for providing us with influenza virus stocks prepared by B. Madeline and S. Aspa, to G. Parnahos-Bacalca, (Fondation Mérieux) for HCV core and E2 proteins, and to J-P. Chauvin (IBDM) for an expert help with electron microscopy.

Author Contributions
Conceived and designed the experiments: FGR CD TFB DO IH. Performed the experiments: FGR CD. Analyzed the data: FGR CD. Contributed reagents/materials/analysis tools: PH TFB. Wrote the paper: IH.

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