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RESEARCH PAPER

HCV J6/JFH1 Tilts the Capability of Myeloid-Derived Dendritic Cells to Favor the Induction of Immunosuppression and Th17-Related Inflammatory Cytokines

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ABSTRACT

Purpose How HCV virus affects the function of dendritic cells (DCs) and their ability to induce CD4+ Tcell response remains not fully understood. This study was done to elucidate the impact of HCV on the function of DCs and on DC's capability to induce CD4+ T-cell response.

Methods Monocyte-derived DCs (MoDCs) were treated with cell-culture HCV (HCVcc). The effects of HCVcc on DC maturation, CD40L-induced DC maturation, and cytokine production and the capacity of DCs to induce Th cytokine production of allogeneic CD4+ T cells were evaluated.

Results HCVcc exposure increased expression of both IL-6 and IL-10 by MoDCs. HCV-exposed MoDCs also selectively facilitated allogeneic CD4+ T cells to further produce Th17-related cytokines interleukin 1 (IL-1), IL-6, and IL-17A. Pretreatment of IL-17A inhibited HCV production in Huh7.5 cells, suggesting that induction of Th17 cells may be beneficial to host anti-HCV immunity. Paradoxically, induction of IL-10 expression and the failure of HCV-exposed MoDCs to facilitate other Th cell development may hinder the anti-viral immunity.

Conclusions This study highlights both the therapeutic potential of IL-17A in treating HCV infection and the cautious consideration of HCV-induced immunosuppression in DC-based therapy.

KEY WORDS CD4+ T cells · dendritic cells · HCV · IL-10 · IL-17A · Th17

INTRODUCTION

HCV, a positive-stranded RNA virus, is a major pathogen causing chronic liver disease. HCV infects approximately 3% of the world's population (1,2). Without effective antiviral treatment, up to 80% of cases will persistently carry HCV and develop chronic liver diseases including hepatitis, cirrhosis, and hepatocellular carcinoma (1,2). Studies have revealed that the role of CD4+ T cells appears to be a critical factor for viral persistence (3,4). Effective CD4+ T-cell response can help patients recover from acute HCV infection (3–5). HCV-specific CD4+ T cells are found only in acute infection and newly resolved patients, not in chronic cases (6), and loss of virus-specific CD4+ T-cell response after acute infection correlates with HCV recurrence in HCV-resolved patients (7). Although cytotoxic CD8 T cells are critical for clearance of HCV infection, sustained HCV-specific CD4+ T-cell response is believed to be indispensable for effective CTL response and ultimate control of the virus (3,8,9).

Dendritic cells (DCs) are the most effective antigen-presenting cells that activate T cells (10,11). DCs can also regulate the types of CD4+ T-cell responses by expressing distinct Th-inducing cytokines (12). Antigen-primed CD4+ T cells are very diverse in function and phenotype, and can be divided into the following subsets: Th1, Th2, Th9, Th17, TFH, and iTregs (inducible regulatory T-cells) (13,14). Th1 cells secrete IFN-γ, IL-2, and tumor necrosis factor-alpha

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(TNF-α) and promote the generation of cytotoxic CD8+ T lymphocytes. Th2, Th17, and TFH cells appear to favor humoral immune responses. iTregs, like natural regulatory T cells, produce IL-10 and TGF-β, inhibiting other Th cells and the CTL response. The Th cells primed by HCV-exposed DCs therefore likely affect the quality and quantity of both cellular and humoral immune responses to HCV infection.

DCs isolated from chronic HCV-infected patients have functional deficits (15–17). Several studies have consistently shown that either HCV proteins or HCVcc *in vitro* alter DC function (18–21). The addition of HCV core and NS3 proteins in cell culture reduces the capacity of DCs to trigger CD4+ T cell proliferation (19–22). HCV envelope glycoproteins E1 and E2 both interact with DC-SIGN molecule on the DC cell surface to mediate viral entry (23,24). HCVcc exposure leads to myeloid DC maturation (25), while it inhibits TLR9-mediated activation of plasmacytoid DCs (26). The effects appear to be associated with the type or strength of toll-like receptor stimuli (25). HCV core exposure inhibits IL-12 expression and thereby the capability of DCs to mount a Th1 response (19). However, the mechanism of how HCV exposure affects the ability of DCs to elicit responses of other Th subsets remains to be further elucidated.

In this study, we cultured MoDCs with HCVcc and investigated the effect of HCV exposure on the capability of DCs to trigger Th differentiation of activated CD4+ T cells. Our results suggest that HCV exposure preferentially facilitates allogeneic CD4+ T cells to produce Th17 cytokine IL-17A and other pro-inflammatory cytokines IL-1, IL-6, and TNF-α. HCV exposure did not affect the production of other Th cytokines. In addition, HCV exposure also elevated autocrine production of IL-10 in MoDCs, likely favoring T-cell tolerance. Our observations, together with those reported previously by others (27), suggest that Th17 induction and dysregulation of cytokine production may contribute to impaired HCV-specific CD4+ T-cell memory and the pathogenesis of HCV infection.

MATERIALS AND METHODS

Isolation of Monocytes and CD4+ T Cells

Peripheral blood mononuclear cells (PBMCs) from anonymous donors were provided by the Red Cross Blood Center of Shanghai. The usage of PBMCs was approved by the ethic committee of the Red Cross Blood Center of Shanghai. Monocytes were separated from PBMCs by density-gradient centrifugation with an isopycnic gradient of Percoll according to a procedure previously reported by others (28). Human CD4+ T cells were purified by separation of PBMCs with the total CD4+ T-Cell Isolation Kit (Miltenyi Biotec) and subsequent depletion of CD25+ cells with magnetic beads (Miltenyi Biotec).

Preparation of HCVcc and Immunofluorescent Staining of Infected Cells

J6/JFH1 chimeric HCVcc was propagated in Huh7.5 cells. Infectious supernatants were filtered through 0.45-um-pore-size filter units, titrated, aliquoted, and stored at −80°C. Highly concentrated virus was prepared using an Amicon Ultra-15 device (100MWCO, Millipore) (29,30). Supernatants from uninfected Huh7.5 cells were also used as a mock control. Accordingly (29,30), infectious virus production was measured by transferring supernatant to Huh7.5 cells, culturing for 48 h, and staining for HCV core expression by immunofluorescence analysis using monoclonal mouse anti-HCV core (C7-50, Abcam) and goat anti-mouse immunoglobulin G conjugated to Alexa Fluor 488 (Invitrogen).

MoDC Culture and Co-Culture with CD4 + T Cells

For generation of MoDCs, monocytes were cultured in RPMI 1640 medium containing 10% fetal calf serum, 50 ng/ml GM-CSF (R&D Systems) and 50 ng/ml IL-4 (R&D Systems) for 5 or 6 days. For studying the effect of HCV on MoDCs, 0.5×10^6 MoDCs were treated with 1.0 multiplicity-of-infection (MOI) HCVcc J6/JFH1 or concentrated uninfected supernatant, which served as mock control, for 4 h. After the treatment, cells were washed 3 times with phosphate buffered saline, and then cultured in fresh plate for an additional 24 h before collection of supernatant and subsequent examination of HLA DR, CD40, and CD83 expression. In some experiments, 10 ng/ml CD40L (eBioscience) was added to the culture. In DC-T cell co-culture, 2×10^4 dendritic cells were co-cultured with 2×10^5 allogeneic CD4+ T cells in 96-well plates. After additional 5 days of culture, the supernatants were harvested for cytokine detection.

Flow Cytometric Analysis of DCs

MoDCs were incubated with human FcγR-binding inhibitor (eBioscience) for 10 min on ice and further incubated with anti-human CD83 PE, anti-human CD40 APC, or anti-human HLA-DR PE-Cy7 antibodies (eBioscience) for another 30 min on ice. Cells were washed twice with staining buffer (phosphate buffered saline containing 0.3% bovine serum albumin and 0.1% sodium azide). The stained cells were re-suspended in staining buffer containing 2.5 μg/ml DAPI (4′,6-diamidino-2-phenylindole) and analyzed on a FACS LSRII analyzer (BD Biosciences). We collected 10,000 cell events for

each sample and analyzed the data further with FlowJo software.

Analysis of Cytokine Production

The supernatant of stimulated DCs and co-cultured cells were analyzed with Human 13plex FlowCytomix Multiplex Kit (eBioscience) according to the manufacturer's instruction. Briefly, 25 μl samples were mixed with 25 μl beads and 50 μl biotin-conjugate. This mixture was incubated for 2 h at room temperature and washed twice. Streptavidin-phycoerythrin (50 μl) solution was added to each sample, incubated for 1 h at room temperature, and washed twice. The stained beads were analyzed on a FACS LSR II analyzer. The acquired data were further analyzed with FlowCytomix Pro 2.4 software (eBioscience).

Quantification of HCV RNA in Infected Huh7.5 Cells

Huh7.5 cells were pretreated with indicated concentrations of IL-17A, IL-17E, or IL-17 F (eBioscience) for various periods of time and infected with HCVcc J6/JFH1 at 0.1 MOI. After 48 h, RNA was extracted using Trizol (Invitrogen). Reverse transcription to obtain cDNA was performed using PrimeScript RT reagent Kit (Takara). Semiquantitative real-time polymerase chain reaction was performed using the SYBR Premix Ex Tag kit (Takara). The primers for the real-time PCR were: HCV NS3 5′-GTGCTGAGGGGGAC TTGG-3′ (sense) and 5′-GCTTGTCCCCGCGTCTC-3′ (antisense); HCV 5′UTR 5′-CCTGGACCCCACCTT CACTA-3′ (sense) and 5′-CCTGCGTCGTAGCACT CAC-3′ (antisense); GAPDH 5′-CTCTGGTAAAGTG GATATTGTTGC-3′ (sense) and 5′-GATTTCCATTGAT GACAAGCTTC-3′ (antisense).

Statistical Analysis

All analyses were performed using GraphPad Prism 5 for Windows (version 11.5; GraphPad Software Inc.). Student's *t*-test was used to determine statistical significance. Difference was considered significant at $p<0.05$.

RESULTS

HCV has an Opposite Effect on Immature and CD40L-Stimulated MoDCs

To determine the effect of HCV on the activation of DCs, we cultured immature MoDCs with HCVcc J6/JFH1 for 24 h and evaluated the expression of activation markers CD40, CD83, and HLA-DR. The presence of HCVcc did not change HLA-DR expression of immature MoDCs when compared with controls. HCVcc induced a small increase of CD40 and CD83 expression on MoDCs (Fig. 1a and b). We also evaluated the effect of HCVcc on CD40L-stimulated MoDCs. As expected, CD40L stimulation did increase the expression levels of HLA-DR, CD40 and CD83. In contrast to the effect on

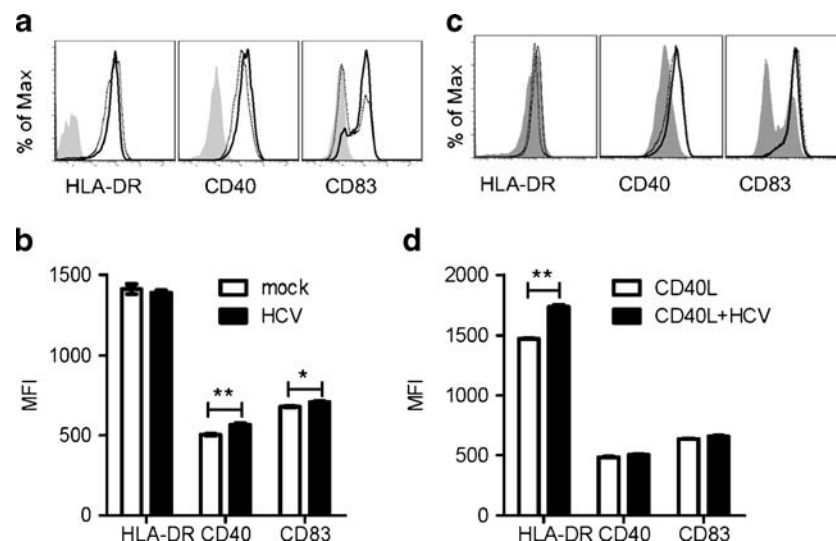

Fig. 1 Effect of HCVcc J6/JFH1 on activation of immature MoDCs. Immature MoDCs were treated with mock or 1.0 MOI of HCVcc J6 JFH in the absence (**a**, **b**) or presence (**c**, **d**) of 10 ng/ml recombinant CD40L, and HLA DR, CD40, or CD86 expression on their cell surface was analyzed with flow cytometry in comparison with isotype control staining. (**a, c**) Representative histogram plots from four independent experiments were shown. Shaded histograms represent isotype control; dashed line, mock treatment; solid line, HCV treatment. (**b, d**) The mean fluorescence intensity (MFI) of HLA DR, CD40, or CD86 expression was shown. Data are presented as mean ± SD of triplicate experiments.
*, $p<0.05$; **, $p<0.01$.

immature MoDCs, HCVcc had no effect on the expression levels of CD40 and CD83 on CD40L-stimulated MoDCs but significantly up-regulated the HLA-DR expression (Fig. 1c and d). Thus, this suggests that HCVcc exposure has only a little but opposite effect on the maturaton of immature *versus* CD40L-stimulated MoDCs.

HCV Preferentially Induces the Production of IL-6 and IL-10 by MoDCs

To study how HCVcc exposure affects cytokine production in MoDCs, supernatants of HCVcc-exposed or unexposed MoDCs which were cultured in the presence or absence of CD40L were measured for cytokine production. IL-12, IFN-γ, IL-2, IL-9, and IL-17A were not detected in any type of MoDC culture (data not shown). MoDCs did express low levels of IL-1, IL-4, and TNF-α, but neither HCV nor CD40L stimulation had any effect on the expression of these cytokines (Fig. 2a-c). MoDCs expressed basal levels of IL-6 and IL-10 before stimulation. HCVcc exposure subsequently led to more than 3-fold increase of both IL-6 and IL-10 expression (Fig. 2d-e). CD40L stimulation did not significantly increase IL-6 production of MoDCs induced by HCV (Fig. 2d). In contrast, CD40L stimulation led to further significantly increase in the IL-10 expression of HCV-exposed MoDCs (Fig. 2e), suggesting that CD40 signaling synergizes with HCV in IL-10 production. HCVcc J6/JFH1 selectively induces IL-6 and IL-10 secretion by immature MoDCs, and CD40 signaling synergizes the induction of IL-10 expression.

HCV-Exposed MoDCs Trigger CD4+ T Cells to Preferentially Produce IL-17A and Other Inflammatory Cytokines

Next, we evaluated the effect of HCVcc on the capability of MoDCs to trigger the Th cytokine production of CD4+ T cells. CD25-depleted CD4+ T cells isolated from healthy donors were co-cultured with allogeneic MoDCs that were cultured for 24 h with HCVcc or mock control. As shown in Fig. 3a-h, exposure of MoDCs with HCVcc did not significantly influence expression of IFN-γ, IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, or IL-22 in allogeneic MoDC/T-cell co-culture supernatants. In other words, HCV exposure did not affect the ability of MoDCs to

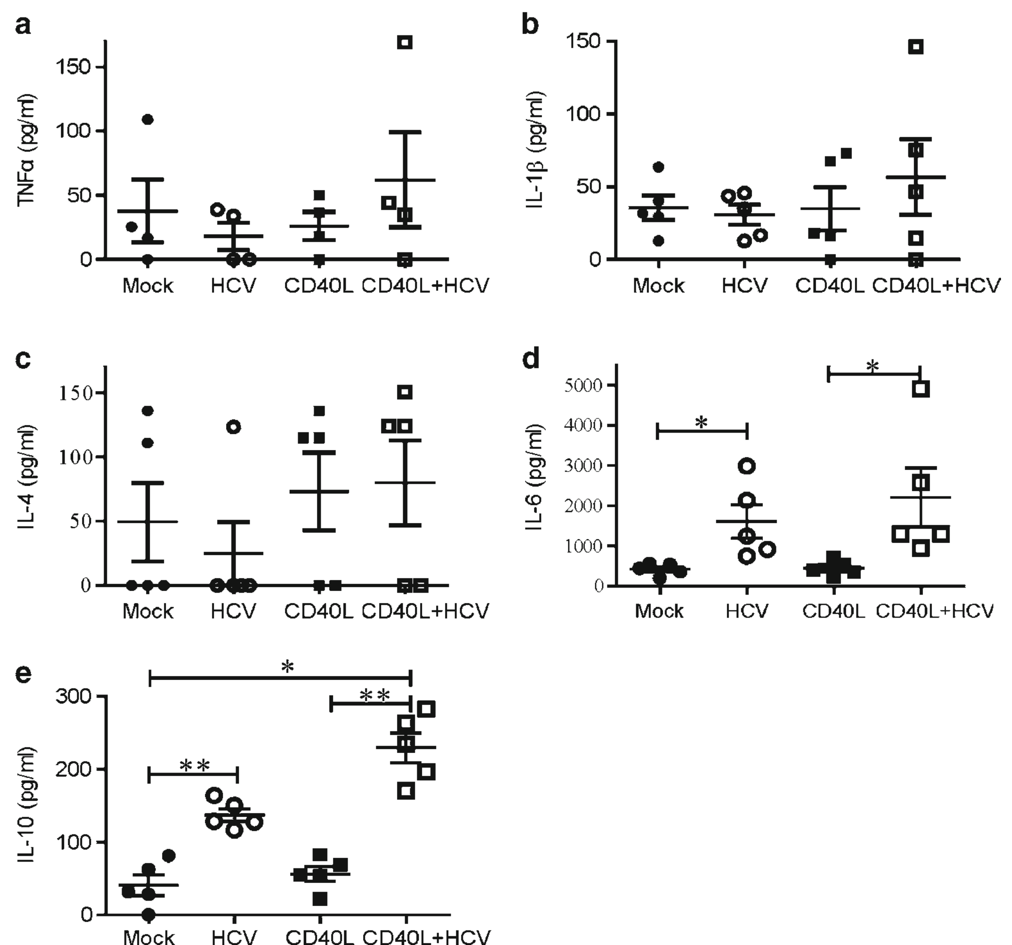

Fig. 2 HCVcc J6/JFH1 exposure selectively promotes IL-6 and IL-10 expression of MoDCs. Immature MoDCs were treated with mock or 1.0 MOI of HCVcc J6 JFH in the absence or presence of 10 ng/ml recombinant CD40L for 24 h. The supernatants were collected from the DC cultures and examined for cytokine expression with multiplex assay. The detectable cytokines including TNF-α (**a**), IL-1β (**b**), IL-4 (**c**), IL-6 (**d**), and IL-10 (**e**) were shown. Each dot represents a sample of CD4+ T cells from one donor. Data are presented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

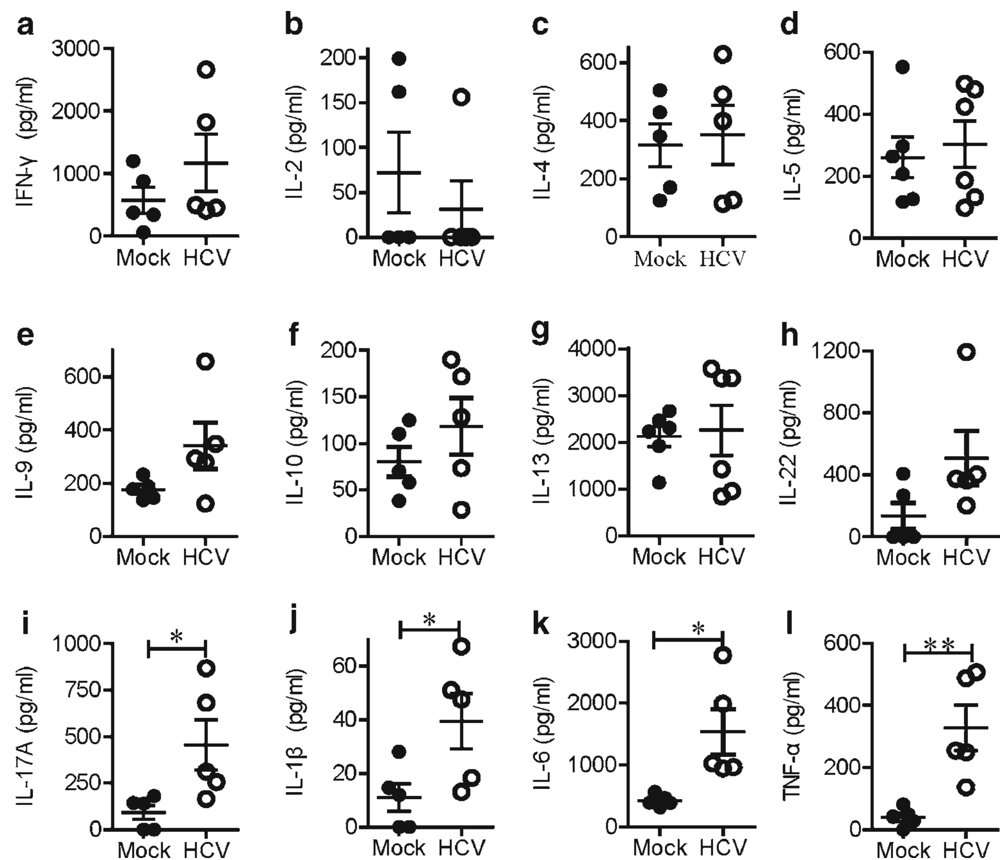

Fig. 3 Exposure of MoDCs with HCVcc J6/JFH1 preferentially facilitate allogeneic CD4+ T cells to produce Th17-related cytokines. (**a–l**) MoDCs that were treated with mock or HCVcc J6/JFH1 and subsequently co-cultured with purified allogeneic CD4+ T cells for 5 days. The supernatants were collected later and assayed for cytokine expression with multiplex. The detectable cytokines were shown. *, $p<0.05$; **, $p<0.01$.

induce the differentiation of allogeneic CD4+ T cells to Th1, Th2, Th9, or Th22 cells. Interestingly, IL-17A in the co-culture of CD4+ T cells with HCV-exposed MoDCs was nearly five times higher than IL-17A in the co-culture with mock-treated MoDCs (Fig. 3i). Consistent with the remarkable increase of IL-17A, Th17-inducing inflammatory cytokines IL-1 and IL-6 were significantly elevated in the supernatants of allogeneic CD4+ T cells that were co-cultured with HCV-exposed MoDCs (Fig. 3j-k). Thus, the elevated expression of IL-1 and IL-6 likely contributes to the increase of IL-17A production by allogeneic CD4 T cells. Similarly, HCV-exposure also significantly increased the production of another inflammatory cytokine, TNF-α (Fig. 3l).

IL-17A, but no Other Th17 Cytokines, Inhibits HCV Production

Considering the preferential increase of Th17 differentiation driven by HCV-exposed MoDCs, we next investigated the influence of Th17 cytokines on HCV replication. Huh7.5 cells were treated with 0, 1, 10, 100, or 1,000 ng/ml recombinant IL-17A, IL-17E, or IL-17F and infected with HCVcc J6/JFH1. Real-time PCR assay using HCV 5' untranslated region revealed that the pretreatment with IL-17F did not affect the amount of HCVcc J6/JFH1 RNA (Fig. 4a), whereas pretreatment at high concentration of IL-17E led to a 20–30% increase in the amount of viral RNA ($p<0.05$, respectively). In contrast, the pretreatment of above with 1 ng/ml IL-17A significantly reduced the amount of HCV RNA in a dose-dependent manner. Maximal decrease of 69% was observed at higher concentration of IL-17A compared to the control cells (Fig. 4a). Immunofluorescence analysis revealed smaller viral foci after IL-17A pretreatment compared to control (Fig. 4b), suggesting that IL-17A may inhibit virus spread. In addition, the inhibition of IL-17A treatment appeared to be time-dependent. Pre-treating Huh7.5 cells with IL-17A 24 h prior to HCVcc J6/JFH1 infection resulted in maximal inhibition of viral titers compared to 48-hour pretreatment or treatment at the time of infection (Fig 4c). Similar data were observed with real-time PCR of NS3 (data not shown). These results indicate that IL-17A potentiates a transient antiviral activity in Huh7.5 cells but does not directly antagonize HCV infection.

DISCUSSION

DCs are the most effective antigen-presenting cells that initiate T-cell response (10,11) and play a central role in governing CD4+ T-cell differentiation. Although both HCV proteins and replicative-form HCV have been

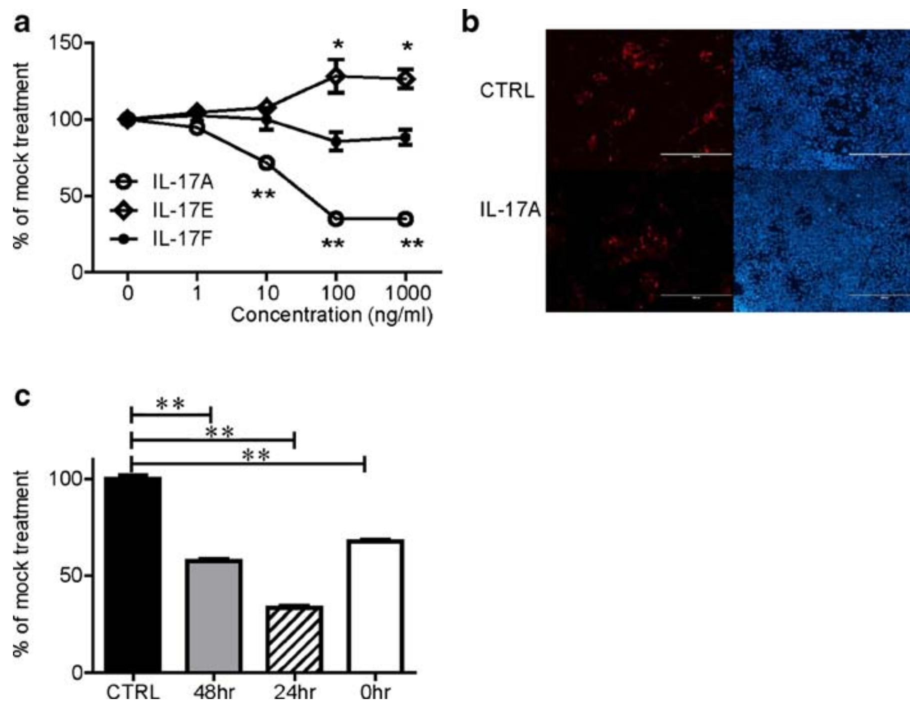

Fig. 4 IL-17A inhibits HCVcc J6/JFH1 replication *in vitro*. (**a**) Hu7.5 cells were cultured with the indicated concentrations of IL-17A, IL-17E, and IL-17F and infected with HCVcc J6/JFH1. The infected cells were harvested after 24 h and the amount of HCV RNA was quantified with real-time PCR assay. Representative data (mean ± standard deviation of triplicate samples) from at least three independent experiments are presented relative to the mock-treated cells. (**b**) Huh7.5 cells which were pretreated with (lower panel, IL-17A) or without (upper panel, CTRL) 100 ng/ml IL-17A were infected with 0.01 MOI HCVcc J6/JFH1. The infected cells were stained with an antibody to core (*red*) and DAPI, a nuclear stain (*blue*). **c** Huh7.5 cells were cultured with 100 ng/ml IL-17A at the indicated time and then infected with HCVcc J6/JFH1. The infected cells were harvested after 24 h and the amount of HCV RNA was quantified with real-time PCR assay. Representative data (mean ± standard deviation of triplicate samples) from at least three independent experiments are presented relative to the mock-treated cells. *, $p<0.05$; **, $p<0.01$.

previously demonstrated to modulate the maturation of DCs and their capability to activate CD4+ T cells, replicative-form HCV promotion of CD4+ T cell differentiation has not been fully understood. Our results suggest that HCV exposure of MoDCs does not affect their ability to promote Th1, Th2, Th9, or Th22 cytokine production in CD4+ T cells. Remarkably, however, the production of Th17 type cytokine, IL-17A was induced. The Th17 stimulation was consistent with increased IL-6 secretion of HCV-exposed MoDCs. In addition, the interaction of HCV-exposed MoDCs with CD4+ T cells also facilitated the production of IL-1β and IL-6, further promoting Th17 differentiation.

Besides IL-1β and IL-6, HCV exposure also enhanced another inflammatory cytokine, TNF-α, in the co-culture of MoDCs and allogeneic CD4+ T cells. Paradoxically, HCV exposure augmented the production of anti-inflammatory cytokine IL-10 by MoDCs. This observation is consistent with the results of a previous study showing that HCV core and NS3 trigger the production of TNF-α and IL-10 in human monocytes (22). IL-1, IL-6, IL-10, and TNF-α are nuclear factor kappa-B-regulated genes (31). HCV core not only activates nuclear factor kappa-B (22) but also phosphorylates signal transducer and activator of transcription 3 through its interaction with gC1qR in human monocytes, macrophages, and dendritic cells (19). Thus, one plausible explanation is that such synergistic signaling, together with other signals, results in the expression of both pro-inflammatory and anti-inflammatory cytokines.

As reviewed previously by Liang *et al.*, different results have been reported with regard to the effects of HCVcc or HCV proteins on the maturation and function of DCs (25). Liang *et al.* found that HCV has different effects on the maturation and stimulatory function of freshly separated myeloid DCs and plasmacytoid DCs to TLR stimulations. The HCV effects are dependent on the type and strength of TLR stimulation (25). This study suggests that HCVcc had a slight but opposite effect on the expression HLA-DR, CD40 and CD83 of immature MoDCs compared to that of CD40L-activated MoDCs. Thus, it is plausible that the discrepant observations may reflect the variation in activation status of a sub-population of DCs, and may represent the complexity of distinct DC sub-populations in patients with various disease conditions.

Th17 cells are frequently found in HCV-infected patients (32–35). However, induction of Th17 cells may have multiple effects on the pathogenesis of HCV infection. We found that IL-17A, but not other Th17 cytokines,

potentiated the capability of host cells to inhibit HCV replication. Accordingly, the increase of virus-specific Th17 response has been found to correlate with spontaneous recovery from recurrent hepatitis C in a liver transplant recipient (35). In contrast, Th17 cytokines together with other inflammatory cytokines may exacerbate liver damage in patients with chronic liver disease (33,36). Th17 cells are less effective in generating memory T cells (37). In addition, dendritic cells in the presence of IL-10 induce T-cell tolerance (38). The IL-10 production of DCs and Th17 induction by HCV may not favor generation of antigen-specific CD4+ T-cell memory, explaining at least partially why chronic HCV-infected patients have little or no detectable antigen-specific memory T cells (3,4). Thus, immunotherapy may be developed aiming at sparing generation of HCV-specific memory T cells from inflammatory cytokine-mediated host damage.

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