Research Article

Enhanced host immune responses in presence of HCV facilitate HBV clearance in coinfection

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A R T I C L E   I N F O

A B S T R A C T

Hepatitis B virus (HBV)/Hepatitis C virus (HCV) coinfection is frequently observed because of the common infection routine. Despite the reciprocal inhibition exerted by HBV and HCV genomes, the coinfection of HBV and HCV is associated with more severe forms of liver diseases. However, the complexity of viral interference and underlying pathological mechanism is still unclarified. With the demonstration of absence of direct viral interplay, some *in vitro* studies suggest the indirect effects of viral-host interaction on viral dominance outcome. Here, we comprehensively investigated the viral replication and host immune responses which might mediate the interference between viruses in HBV/HCV coinfected Huh7-NTCP cells and immunocompetent HCV human receptors transgenic ICR mice. We found that presence of HCV significantly inhibited HBV replication *in vitro* and *in vivo* irrespective of the coinfection order, while HBV did not affect HCV replication. Pathological alteration was coincidently reproduced in coinfected mice. In addition to the participation of innate immune response, an involvement of HCV in up-regulating HBV-specific immune responses was described to facilitate HBV clearance. Our systems partially recapitulate HBV/HCV coinfection and unveil the uncharacterized adaptive anti-viral immune responses during coinfection, which renews the knowledge on the nature of indirect viral interaction during HBV/HCV coinfection.

1. Introduction

Coinfection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is not rare because of their common route of transmission (Jamma et al., 2010), which could be established by simultaneous co-transmission or by superinfection, defined as a scenario when one virus is transmitted to patients chronically infected by another virus (Chen et al., 2010). In clinics, various coinfection fashions lead to different virus-dominance outcomes and persistence of infection (Raimondo et al., 2006; Matsuoka et al., 2008; Mavilia and Wu, 2018; Marot et al., 2017). Spontaneous clearance of HBsAg and even HBV DNA in patients was reported with high anti-HCV seropositivity in HBV chronically infected patients, indicating that HCV superinfection suppresses or terminates the HBsAg carrier state (Chu et al., 2002; Sheen et al., 1992; Liaw et al., 1997). Active HBV replication was also reported to inhibit HCV infection, with a consequent shorter duration of HCV viremia and consequently a lower

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level of antibody response to HCV (Zarski et al., 1998; Jamma et al., 2010; Chu et al., 2002). However, even though the existence of reciprocal interference between HBV and HCV has been intensively demonstrated, chronic coinfection has been demonstrated contributing to severer diseases compared to HBV or HCV monoinfection does (Raimondo et al., 2006; Zampino et al., 2015). Moreover, the heterogeneity of coinfection and complexity of viral outcome varies the efficacies as well as the standard of interferon (IFN)-based or/and direct-acting antivirals (DAAs) treatments for HBV/HCV infection (Konstantinou and Deutsch, 2015; Sagnelli et al., 2017). A growing number of cases reporting the reactivation of HBV replication in coinfected patients after HCV clearance with DAAs further confirm the interaction between viruses and imply that the treatments might need be adjusted by cases (Blackard and Sherman, 2018; Wahle et al., 2015). Therefore, clarifying the nature of the interaction between viruses is critically important for understanding the coinfection outcomes and giving advises for disease treatment (Roy et al., 2020; Gish, 2017; Sagnelli et al., 2017; Wiegand et al., 2015).

Most previous coinfection studies based on heterologous over-expression of viral proteins in cell lines or mono-infected animal models yielded conflicting results because of the incomplete recapitulation of virus lifecycles (Chen et al., 2003; Zhu et al., 2012; Schuttler et al., 2002). Subsequently, three representative *in vitro* studies with cells supporting viruses lifecycles by Bellecave, Eyre and Yang independently showed that HBV and HCV do co-replicate in the same cells but without overt direct interference (Bellecave et al., 2009; Eyre et al., 2009; Yang et al., 2014), which suggests the probability of the indirectly mediated viral interference observed in patients by innate and/or adaptive host immune responses (Bellecave et al., 2009).

Induction of IFN by one virus to suppress the other virus's replication was usually considered as an important indirect interference mechanism during coinfection *in vivo* (Heim and Thimme, 2014). Compared to the stealthy character of HBV that escapes activation of the IFN pathway during the infection, HCV was found activating the RIG-I-like helicase pathway and inhibiting HBV infection in primary human hepatocytes as well as humanized-liver mice (Murai et al., 2020). That the diminishment of IFN responses induced by HCV after successful DAA treatment reactivated HBV replication in patients also confirmed the importance of IFN responses in suppressing HBV during coinfection (Chen et al., 2010; Hiraga et al., 2009). However, a more limited response of HBV to IFN therapy and a resurgence of HBV after the IFN therapy suggest that additional HBV-specific adaptive immune responses take more important roles in determining the eradication of HBV in coinfection.

It's known that exhaustion of CD^{8+} T cell response accounts for the chronic infection of HBV, while the robust specific CD^{8+} T cell responses eradicates viruses during acute infection (Boni et al., 2007; Urbani et al., 2005; Sagnelli et al., 2017). However, as mouse sodium taurocholate co-transporting polypeptide (NTCP) does not support HBV entry into mouse liver cells, nor the supplementation of human NTCP into murine cell-lines or primary human hepatocytes (PHH) renders them susceptible to HBV infection, a robust immunocompetent model for chronic HBV infection does not exist (Ni et al., 2014; Li et al., 2014). While the low throughput, intra-experimental variability, high cost and blunting antiviral immunity in human hepatocytes chimeric mice hamper the authentical and systematic study of viral interplay as well as immune responses to coinfection, especially the role of host adaptive immune responses in determining the viral dominance and ultimate disease outcomes in coinfection. Here we modeled HBV/HCV coinfection in Huh7-NTCP cell lines which are susceptible to HBV and HCV virions infection and HCV receptors (CD81 and occludin) transgenic immunocompetent mice (C/O^{Tg}) which support persistent infection with HCV particles collected from cell cultures (HCVcc) or chronically infected patients (Chen et al., 2014). Our systems recapitulate HBV/HCV coinfection and immunity in an economical small animal model and demonstrates host immune responses' involvement, especially the previously undescribed adaptive immune responses, in determination of viral infection outcome, which reveals and helps us understanding the role of viral-host interaction during coinfection.

2. Materials and methods

2.1. Plasmids, cell culture, and viral inoculation

HBV cell-cultured particles (HBVcc) were collected and purified from the supernatant of HepG2.2.15. HCVcc were purified from the supernatant of Huh7.5.1 cells infected with HCV J399EM, derived from JFH-1 (genotype 2a) with the insertion of eGFP at the C-terminal of NS5A protein. HCV titration was performed by endpoint dilution assays (EPDA) using 50% tissue culture infective dose ($TCID_{50}$) as the readout in Huh7.5.1 cells (Han et al., 2009). HBV replication-competent plasmid pAAV-1.2HBV was kindly provided by Prof. Pei-Jer Chen (National Taiwan University) (Huang et al., 2006). HCVcc/J399EM ($TCID_{50} = 1 \times 10^7$/mL) and 10 µg pAAV-1.2HBV were inoculated into male C/O^{Tg} mice by tail-vein injection and hydrodynamic injection to establish HBV/HCV coinfection (Huang et al., 2006; Yang et al., 2002).

2.2. HBVcc/HCVcc coinfection of Huh7-NTCP and immunofluorescent staining (IF) assay

Huh7-NTCP cells that stably express the HBV functional receptor-human NTCP were coinfected with HBVcc (cell-cultured) and HCVcc as described in Fig. 1A (Zhao et al., 2018). Immunofluorescent staining data of the HBV core protein with anti-core (Dako, CA, US) and imaging of co-expression of HBV core and HCV NS5A-eGFP were collected using a confocal microscope (Leica, CA, US).

2.3. Enzyme-linked immunosorbent assay (ELISA) and western blotting

HBV antigens and antibodies were diluted 10-fold and tested by ELISA according to the manufacturer's protocols (Chen et al., 2014; Cao et al., 2014). HCV core suspension was prepared from HCVcc and used for precoating 96 well-plates (Thermo Fisher Scientific, MT, US) with 1 mg per well for following HCV core antibody ELISA test under the protocol modified from a previous publication (Welker et al., 2000). Anti-NS3 (Abcam, cat# ab65407), anti-Core (Abcam, cat# ab2740), and anti-beta-actin (Santa Cruz, cat# sc-47778) were used for Western blot tests.

2.4. Detection of liver HBV DNA, HBV or HCV RNA, serum HBV core-associated DNA, and gene expression levels in the liver with southern blot and RT-qPCR

HBV DNA, RNA, and HCV RNA were extracted and detected as described previously (Liu et al., 2017; Han et al., 2009). For extraction of HBV core-associated DNA, cell supernatant or plasma were lysed with 10 mmol/L $MgCl_2$ and 100 µg/mL DNase I (Sigma-Aldrich, St. Louis, MO) and maintained at 37 °C for 0.5 h. The DNase I digestion was stopped using 25 mmol/L EDTA (pH 8.5). Then, the samples were further digested with 5 mg/mL proteinase K (Qiagen, CA, US) and 1% SDS at 55 °C for 2 h. The HBV replicative intermediates were extracted with a phenol-chloroform mixture (1:1 ratio) and subjected to quantitative real-time PCR as described previously. The gene primers used for RT-qPCR are listed in Supplementary Table S1. The levels of gene expression were normalized to beta-actin or GAPDH.

2.5. Immunohistochemistry staining (IHC) and hematoxylin and eosin (H&E) staining

H&E staining and IHC staining of liver tissue were performed as described previously (Liu et al., 2017; Su et al., 2017). The arrows showed the protein expression of positive cells or moderate micro-vesicular steatosis. The percentages of microvesicular steatosis area and positive cells were analyzed with ImageJ (NIH). Scale bar = 100 µm.
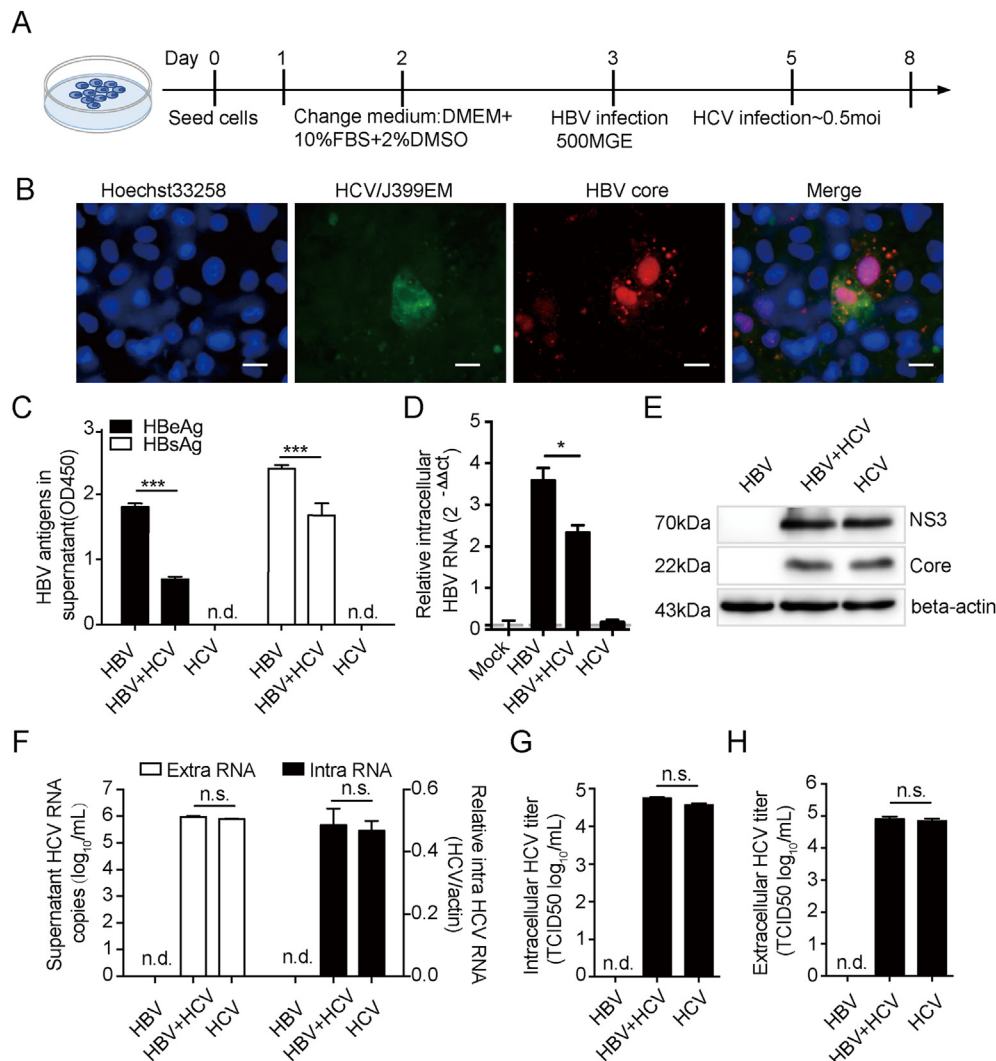
Fig. 1. HCV inhibits HBV replication while HBV does not affect HCV replication in coinfected Huh7-NTCP cells. **A** Schematic presentation of experiment design. Huh7-NTCP cells were treated DMEM medium containing 10% FBS and 2% DMSO before HBVcc infection which was followed by HCVcc infection to establish coinfection *in vitro*. **B** HBV core and HCV NS5A expressions. Scale bar = 100 μm. **C** Supernatant HBeAg and HBsAg. **D** Intracellular HBV RNA was tested by relative quantification using RT-qPCR. Gray dot dash indicates the baseline of the test. **E** HCV NS3 and core protein. **F** Extracellular and intracellular HCV RNA. **G** Intracellular and **H** extracellular HCV titers. The statistical significance is calculated using the two-tailed, unpaired *t*-test. *, $P < 0.05$; ***, $P < 0.001$; n.s., not significant ($P \geq 0.05$); n. d. not detected.

2.6. ELISpot assay, intracellular cytokine staining (ICS), and flow cytometry

Intrahepatic leukocytes and splenocytes were isolated and detected with ELISpot and ICS as described previously (Liu et al., 2017). Peptides corresponding to HBsAg T cell epitopes ($K^b/S_{190-197}$, VWLSVIWM; K^d/S_{28-39}, IPQSLDSWWTSL; K^b/S_{19-33}, and FFLLTRILTIPQSLD) and the HBcAg T cell epitopes (K^b/C_{93-100}, MGLKIRQL; K^d/C_{87-95}, NYVNTNMGL; $K^b/C_{129-140}$, and PPAYRPPNAPIL) were used for restimulation. Flow cytometry data were acquired on LSRII flow cytometers (BD, NJ, US) and analyzed using FlowJo (Tree Star, NJ, US) (Liu et al., 2017).

2.7. Serum chemistry test and multiple cytokines detection

Serum collected at the indicated time points was subjected to serum chemistry test by automated Blood Chemistry Analyzer (BioSino Biotech, Beijing, China). Multiple cytokines were tested with LEGENDplex™ Mouse multiplex kits (Biolegend, CA, US) as described previously (Chen et al., 2014).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA). Significant differences were analyzed using a two-tailed unpaired *t*-test. The two-way ANOVA, followed by Bonferroni's test, was used to determine the differences in multiple comparisons. *P*-values were calculated, and statistical significance is reported as highly significant using * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$) or n.s., not significant ($P \geq 0.05$). Data are presented as the mean ± standard deviation.

3. Results

3.1. HCV inhibits HBV replication in Huh7-NTCP cells

Given that most pervious HBV/HCV coinfection research were based on viral protein-overexpression system, we co-inoculated Huh7-NTCP cells with HBVcc and HCVcc as *in vitro* model to closely mimic natural coinfection (Zhou et al., 2017). We superinfected HBVcc-infected Huh7-NTCP cells with

HCVcc as described in the schematic diagram (Fig. 1A). The immunofluorescence staining of HBV core and HCV NS5A proteins revealed the effective coinfection of HBVcc and HCVcc in the same Huh7-NTCP cells (Fig. 1B), which is in agreement with a previous clinical report (Rodriguez-Inigo et al., 2005). Although the coinfection rate is low to 1%–3%, the HBV and HCV mono-infection rates are around 10% and 5%–8%, respectively. The following virological tests further showed that HCV significantly decreased the levels of supernatant HBV e, surface antigens (HBeAg and HBsAg) and intracellular HBV RNA intermediates (Fig. 1C and D) in coinfected cells compared to those in cells with HBV alone. While HCV protein expressions (NS3 and core), intracellular and extracellular RNA, and infectious viral particles were comparable between the coinfected- and mono-infected cells (Fig. 1E–H). These results suggest that HCV inhibits HBV replication, while

HBV does not affect HCV replication in current *in vitro* system. It's consistent with the findings in another two *in vitro* coinfection systems with varying coinfection rates which were independently reported by Cheng and Zhang et al. (Cheng et al., 2020; Zhang et al., 2021). Taken together, the results suggest that the infection rate of each virus seems taking minor role in determining the coinfection outcomes which implies the possibility of suppression of HCV on HBV mediated by host-viral interplay.

3.2. HCV suppresses HBV replication in immunocompetent transgenic ICR mice

To confirm our hypothesis that the viral interference is mediated by the indirect viral-host interaction, we further recapitulated and studied
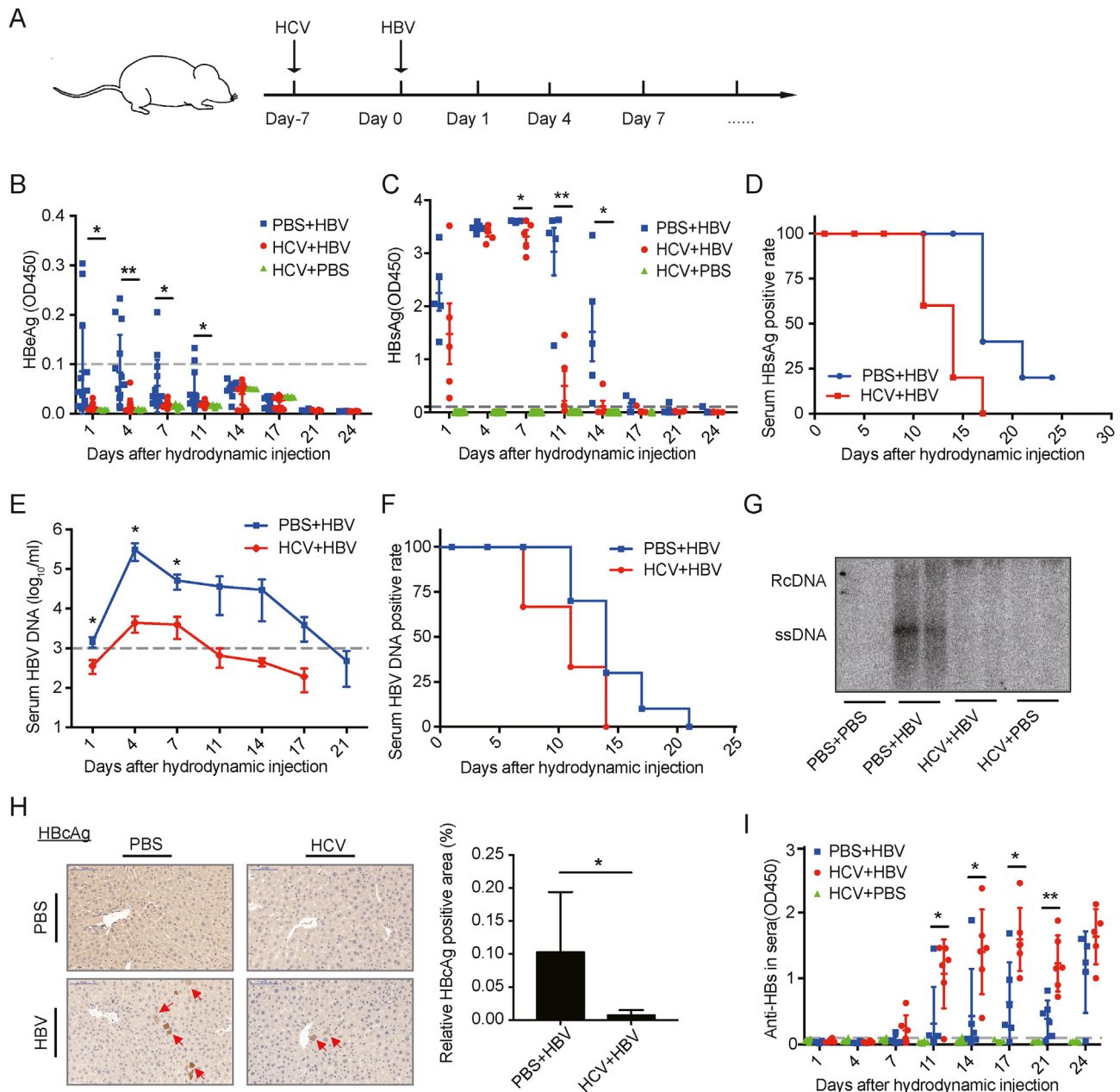

Fig. 2. HCV inhibits HBV replication in immunocompetent transgenic ICR mice. **A** Schematic presentation of the experiment design. ICR transgenic mice were infected with HCVcc followed by pAAV-1.2HBV hydrodynamic injection at 7 days post the HCVcc infection. HBV replication was detected at indicated time points. Serum HBeAg (**B**), HBsAg (**C**), HBsAg positive rates (**D**), Serum HBV core-associated DNA (**E**), and Serum HBV DNA-positive rates (**F**) were analyzed at indicated time points. **G** HBV DNA intermediates in the liver at 7 dpi. **H** HBV core expression in liver and percentages of HBcAg-positive area (right panel). Scale bar = 100 μm. **I** The dynamic of anti-HBs antibody levels in sera. Four to six mice in each system of each batch were analyzed. The statistical significance is calculated using the two-tailed, unpaired *t*-test. *, $P < 0.05$; **, $P < 0.01$.

HBV/HCV coinfection in immunocompetent HCV receptors transgenic ICR mice. Firstly, HBV replication after hydrodynamic injection of the pAAV-1.2HBV plasmid, which is commonly employed for HBV research in immunocompetent mice (Liu et al., 2017; Huang et al., 2006), into wild-type ICR mice was pre-tested. The dynamic levels of serum HBeAg and HBsAg as well as core-associated HBV DNA demonstrated that pAAV-1.2HBV generates acute HBV infection in ICR mice (Supplementary Figs. S1A–D), as it does in BALB/c mice (Yang et al., 2002). The ecotropic expression of HBV core and S protein in hepatocytes but not in other organs detected with IHC further suggested faithful recapitulation of acute HBV infection in this model (Supplementary Figs. S1E–F).

As HCV generates chronic infection in C/O^{Tg} ICR mice, a hydrodynamic injection of pAAV-1.2HBV after 7 days of HCV pre-infection mimicking HBV superinfection in ICR mice was chosen for the following study (Fig. 2A). Mouse blood or tissues were collected at indicated time points for virological and immunological tests. The results showed that expression of serum HBeAg was high from the first day post hydrodynamic injection (dpi) in control mice and declined afterwards but was undetectable in coinfected mice throughout the experiment (Fig. 2B). Likewise, serum HBsAg was negative in approximately 20% (4/19) of HBV/HCV coinfected mice but positive in all control mice before the clearance, which indicates the suppressed HBV infection in coinfected mice. In HBsAg positive coinfected-mice, the average serum HBsAg level was lower than that in HBV control mice and followingly increased to the comparable peak level, but then decreased sharply and quicker than that in the control mice at 11 dpi (Fig. 2C). Consequently, the serum HBsAg clearance happened earlier in coinfected mice (Fig. 2D). Likewise, the serum HBV core-associated DNA copies were detected above the measurement baseline in all control mice, but only in 64% (7/11) of HBsAg-positive coinfected mice. HBV DNA average levels were significant lower in coinfected mice (Fig. 2E) and eliminated faster compared with those in HBV mono-infected mice (Fig. 2F). In liver, the lower levels of HBV DNA intermediates and HBcAg expression further confirmed a limited HBV replication with the presence of HCV (Fig. 2G and H). The earlier appearance and robust titers of anti-HBsAg antibodies were determined (Fig. 2I), explaining the lower and faster clearance of HBsAg in coinfected mice. Taken together, the virological and antibody tests demonstrate that HCV suppresses HBV replication and accelerates HBV clearance which is partially mediated by antibody response.

To study the viral interaction in various coinfection orders, another two coinfection strategies were investigated, namely 1) hydrodynamic injection of the mixture of pAAV-1.2HBV and HCVcc and 2) superinfection of HBVcc-infected mice with HCVcc. The dynamic serum HBsAg and anti-HBsAg antibodies levels were monitored to denote HBV replication in all mice. For the simultaneous injection strategy which mimics simultaneous coinfection, the HBsAg levels in coinfected mice were undetectable whereas positive in all HBV mono-infected mice. Accordingly, anti-HBs antibody response was barely detected at one week post infection in coinfected mice but robust in control mice (Supplementary Figs. S2A–C). In HCV superinfection strategy, serum HBsAg levels in coinfected mice were comparable to those in the control mice at peak but decreased faster afterwards. Consistently, anti-HBs antibody levels were higher in coinfected mice than those in control group (Supplementary Figs. S2D–F). Taken together, all strategies suggest that HCV inhibits HBV replication irrespective of coinfection order in immunocompetent mouse model.

3.3. HBV does not affect HCV replication in immunocompetent transgenic ICR mice

HBV has also been reported as the dominant virus and inhibits HCV replication in some coinfected patients (Guido et al., 1998). To detect the effect of HBV on HCV, we compared HCV replication in coinfected mice with that in HCV mono-infected mice. The detection of HCV NS3, core protein and serum as well as intrahepatic HCV RNA levels indicated the well replication of HCV in both coinfected and control mice (Fig. 3A).

Serum HCV RNA levels in coinfection and HCV control groups were comparable at 1 and 7 dpi (Fig. 3B). HCV RNA levels in mice decreased gradually in the first two weeks post infection, and afterwards remained stable in the chronic infection state as described previously (Chen et al., 2014). No change in HCV NS5A or core protein expression or intrahepatic HCV RNA levels was observed after HBV superinfection (Fig. 3A–C). Consistently, serum anti-HCV core antibody levels showed no significant difference between two groups, either (Fig. 3D). Therefore, we conclude that superinfection of HBV has no significant effect on HCV replication or infection state in HBV/HCV-coinfected ICR mice.

3.4. Pathological alterations are induced in the liver of HBV/HCV-coinfected but not control mice

Despite the reported conflicting viral outcomes due to the various coinfection conditions, chronical HBV/HCV dual infection has been considered as a serious determining factor to more severe forms of liver diseases (Sagnelli et al., 2017), characterized by an increased serum alanine aminotransaminase (ALT) (Sagnelli et al., 2014). However, the comparable levels of serum ALT in all mice groups indicated the absence of severe diseases like fibrosis or cirrhosis in liver, consistent with previous report that pronounced fibrogenesis was mainly observed at 4 months post infection (mpi) in HCV-chronically infected ICR transgenic mice (Chen et al., 2014) (Fig. 4A). Also, since the degree of ALT alteration is always shown in a range, limited to guide the severity of the liver condition, we next graded the liver function by detecting the production of albumin (ALB). The data showed that the serum ALB levels in coinfected mice dropped down to lowest point of the observation course at 7 dpi, and afterwards increased back to comparable levels to non-infected mice (Fig. 4B). The falling down of ALB levels reflected a transient liver impairment which restored to normal later. The following observation of moderate microvesicular steatosis confirmed the presence of histopathological alteration in coinfected mice but not in mono-infected mice. Concomitant with rebound of serum ALB, the microvesicular steatosis recovered after HBV clearance (Fig. 4C). By contrast, HBV or HCV alone did not induce histopathological damage in the liver during the observation period of three months except for mild leukocytic infiltration. These data indicated that the transient damage to liver was presumably a consequence of coinfection of HBV and HCV, in accordance with previous reports about more severe liver diseases in coinfected patients regardless of the final viral outcomes (Sagnelli et al., 2014). The absence of obvious histopathological manifestation in HBV mono-infected mice may attribute to the acute infection of HBV which was cleared before the severe liver damage. Collectively, these data demonstrate that HBV/HCV coinfection induces liver pathological alterations which are not observed in HBV or HCV mono-infection.

3.5. HCV activates the innate immune response in HBV/HCV coinfected mice

Accumulating evidence has suggested a causal relationship between the host innate immune response and viral coinfection outcome. To clarify the host immune responses to HBV/HCV coinfection, we next examined the mRNA levels of IFNs, IFN-stimulated genes (ISGs) and proinflammatory cytokines in mouse liver at the first phrase after coinfection. The color changes in heat-maps showed that cytokine expressions were strongly activated by HCV infection but not HBV mono-infection in ICR mice. While the cytokine expressions in HBV/HCV coinfected mice were higher than those in HBV mono-infected mice, and comparable to those in HCV mono-infected mice at day one, which is consistent with previous report (Cheng et al., 2020) (Fig. 5A). The increases in ISG and proinflammatory gene expressions at day seven post infection indicated the lasting of IFN response in the immunocompetent mice (Fig. 5B). Coincidentally, the levels of IFN-γ and other proinflammatory cytokines (TNF-α, IL-23, IL-17a) in serum increased and were higher in coinfected mice than those in control mice (Fig. 5C). Our data
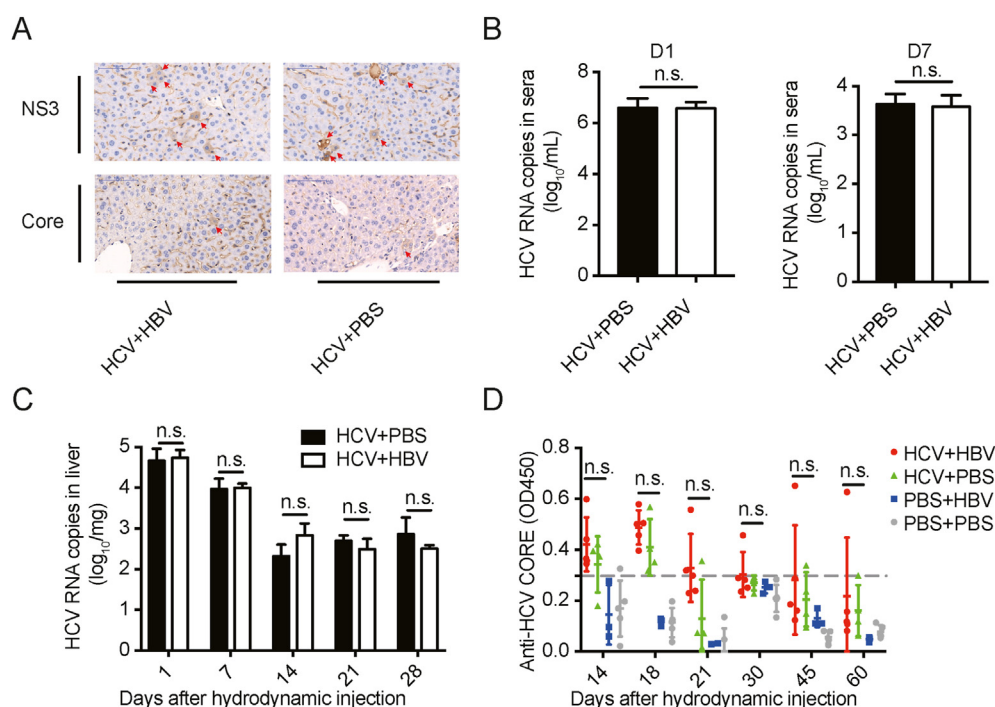
Fig. 3. Presence of HBV has no effect on HCV replication *in vivo*. ICR transgenic mice were infected with HCVcc followed by pAAV-1.2HBV hydrodynamic injection at 7 days post the HCVcc infection. HCV replication was detected at indicated time points. **A** HCV NS3 and core protein were detected with IHC at 7 dpi. Scale bar = 100 μm. Levels of serum HCV RNA at 1 dpi and 7 dpi (**B**) and HCV RNA in the liver at different time points (**C**). **D** Levels of anti-HCV core antibodies in serum. The statistical significance is calculated using the two-tailed, unpaired *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; or n.s., not significant ($P \geq 0.05$).
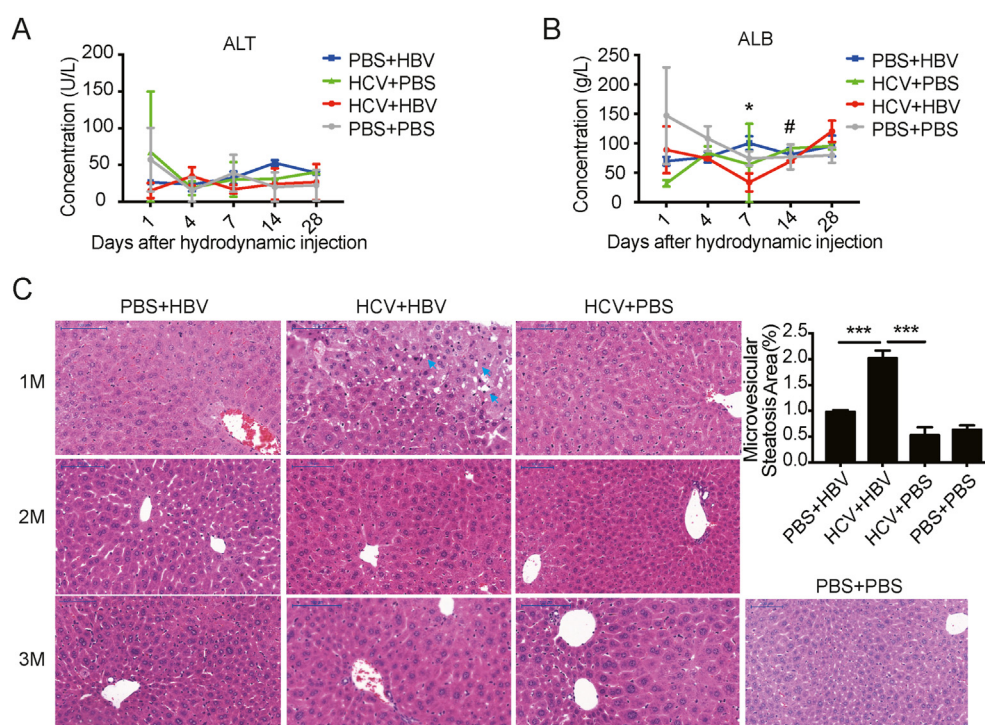

Fig. 4. Pathological alterations are induced in the liver of HBV/HCV coinfected mice. Mouse livers were collected from HBV/HCV coinfected and control ICR transgenic mice at 1 M (month), 2 M, 3 M post-HBV superinfection. Levels of ALT (**A**) and ALB (**B**) in sera. **C** H&E staining of liver sections. The livers of PBS control mice were obtained at 1 M post coinfection. The relative area of microvesicular steatosis of 1 M mono- or coinfected-livers were analyzed in three mice for each group (right panel). Scale bar = 100 μm. Five random views per section were chosen and analyzed. The statistical significance is calculated using the two-tailed, unpaired *t*-test. PBS + HBV *vs* HCV + HBV, * ($P < 0.05$); HCV + PBS *vs* HCV + HBV, # ($P < 0.05$).

demonstrate that the activation of innate immune response by HCV probably contributes to the limitation of HBV replication in coinfected mice.

3.6. HCV enhances anti-HBV-specific CTL response

Because HCV infection induces robust innate immune response, including the increase of IFN-γ and a greater anti-HBV humoral immune response in HBV/HCV coinfected mice, we hypothesized that HBV specific CTL responses might also be boosted by the existence of HCV. To address this, the intrahepatic lymphocytes and splenocytes were isolated at 28 dpi and *ex vivo* re-stimulated with the pools of HBV epitope peptides derived from HBsAg or HBcAg. Secretion of IFN-γ was detected with ELISpot. The results showed that the presence of HCV significantly increased the IFN-γ production of intrahepatic lymphocytes from coinfected mice compared with HBV mono-infection control mice (Fig. 6A). A similar increase in IFN-γ secretion was also found in splenocytes (Fig. 6B). To further characterize the cell populations simultaneously producing various cytokines after restimulation with HBV peptides *ex vivo* in the spleen, the multiparameter staining of splenocytes with both the surface phenotypic markers and intracellular cytokines was performed and analyzed by flow cytometry. The results showed that the percentages of single cytokine (IFN-γ, TNF-α or IL-2) producing CD4$^+$ and CD8$^+$ T cells were much higher in coinfected mice than in HBV mono-infected control mice (Fig. 6C–D and Supplementary Figs. S3A–C). Further analysis on the percentages of multiple cytokines-producing CD4$^+$ and CD8$^+$ T cells, which simultaneously produce two or three of these cytokines, consistently showed a significant increase in the HBV/HCV-coinfected mice, indicating that more splenic CD4$^+$ and CD8$^+$ T cells from coinfected mice were polyfunctionally activated to produce cytokines (Fig. 6E–F and Supplementary Figs. S3D–F). Thus, the results

demonstrate that the presence of HCV enhances the cell-mediated specific immune responses to HBV in immunocompetent mice. Taken together, our model recapitulates HBV/HCV infection and demonstrates that HCV enhances host immune responses, especially the HBV adaptive immune response, to inhibit HBV replication and facilitate HBV clearance. This study reveals the previously uncharacterized specific immune responses to HBV during coinfection, which fills gaps in the knowledge on host immune responses to coinfection and prompts us to reconsider the role of HCV in HBV/HCV coinfection.

4. Discussion

Here, we mimicked HBV/HCV natural coinfection in Huh7-NTCP cells and immunocompetent mice. We found that HCV inhibits HBV replication regardless of coinfection order. In line with our findings, HBV was proved as inhibited by coinfecting with HCV in a new cell line HepG2-NTCP-CD81-Mir122 developed by Zhang et al. which supports robust coinfection of HBV and HCV (Zhang et al., 2021). Likewise, Cheng et al. also showed the suppression of HCV on HBV irrespective of the coinfection sequence in coinfected PHH. Treating HCV with DAAs induced rebounded HBV replication (Zhang et al., 2021; Cheng et al., 2020). The collective data of different systems, including both high HBV and HCV infection (HepG2-NTCP-CD81-Mir122) or high HBV but low HCV infection (PHH) as well as our both low HBV and HCV infection systems (Huh7-NTCP), showed the same conclusion that presence of HCV suppresses HBV replication during coinfection. The various infection rates of each virus in each system seem taking minor role in determining the coinfection outcome, which imply the high possibility that HCV suppress HBV replication through indirect interference.

On the other hand, recent studies in humanized *cDNA-uPA/SCID* mice and superinfection of HBV in chronically HCV infected chimpanzees
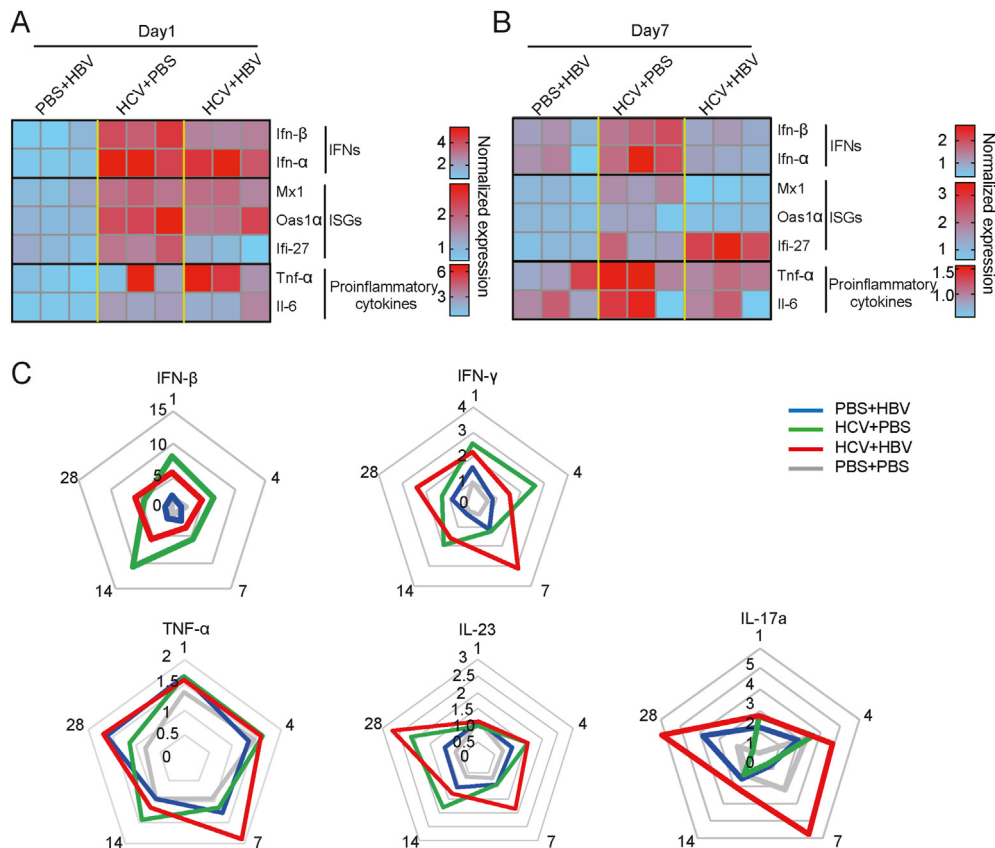

Fig. 5. Changes of innate immune response-related cytokines after coinfection. **A–B** Heatmaps show the RT-qPCR detection of mRNA levels of *IFN-α*, *IFN-β* and ISGs *Mx1, Oas1a* and *Ifi-27* as well as inflammatory genes *Tnf-α, Il-6* in liver; three mice in each group. **C** Levels of serum cytokines IFN-β, IFN-γ, TNF-α, IL-23, IL-17a at 1, 4, 7, 14, 28 days post infection were shown in radar plots as a fold change, which were calculated by dividing the baseline levels (day 0).
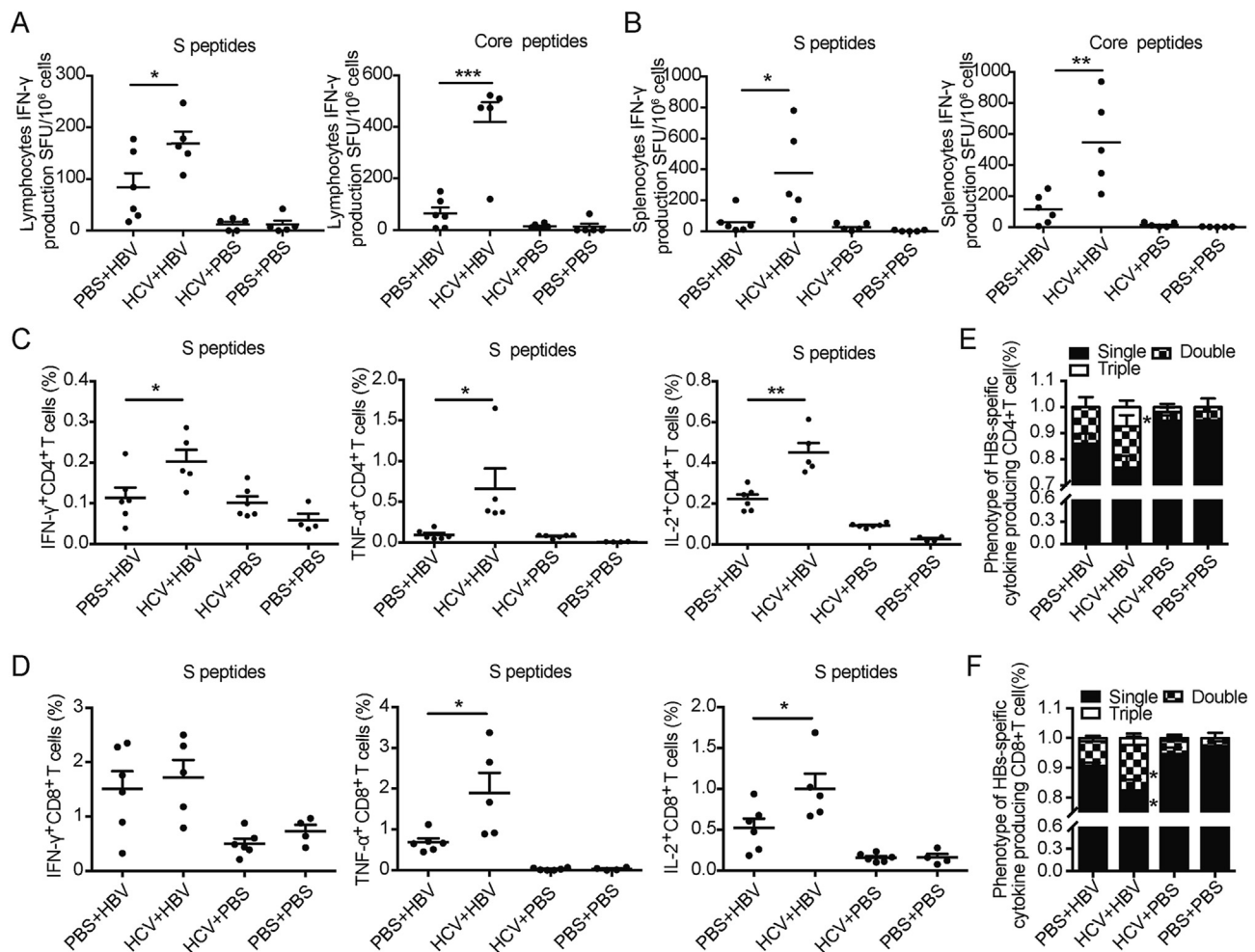
Fig. 6. HCV enhances HBV-specific CTL responses in the liver and spleen. The IFN-γ secreting of intrahepatic lymphocytes (**A**) and splenocytes (**B**) after HBsAg- or HBcAg-derived peptides *ex vivo* restimulation were detected with ELISpot. SFU: spot forming units. The frequencies of IFN-γ$^+$ or TNF-α$^+$ or IL-2$^+$ CD4$^+$ T cells (**C**) and CD8$^+$ T cells (**D**) after *ex vivo* restimulation by HBsAg-derived peptides were detected by intracellular staining followed by flow cytometry analysis. Quantitative analysis of the percentages of one or two or three kinds of cytokines (IFN-γ/TNF-α/IL-2) simultaneously producing cells within CD4$^+$ (**E**) or CD8$^+$ (**F**) T cell population from the spleen. The statistical significance is calculated using the two-tailed, unpaired *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

demonstrated that presence of HBV did not change the HCV viremia significantly, consistent with the findings in our systems as well as most clinical observations which are more frequently characterized by an inhibition of HBV replication exerted by HCV (Chu and Lee, 2008; Cheng et al., 2020; Wieland et al., 2014). Except for the immune/cytokine response, the viral genotype and viral load may also contribute to the outcome of viral dominance. For example, inhibitory effect of HCV was reported to be genotype-dependent and being more evident in the case for genotype 1 in clinics (Shih et al., 1995). As described in previous reports and our study, HCV infection triggers activation of innate immune response which we reason contributing to the suppression of HBV replication. A recent study demonstrated that some secreted soluble factors expressed by HepaRG cells in the presence of IFN-α could block HBV entry to hepatocyte by binding to heparan glycosaminoglycans during the virion attachment step (Xia et al., 2017). Also, Cheng et al. confirmed that HCV infection triggered IFN response and subsequently inhibited HBV replication in PHH as well as in humanized mice. Blocking ISGs expression supports rebound of HBV replication (Cheng et al., 2020). However, as these data were obtained from cells or immune response blunted humanized mice, HBV specific immune responses were uncharacterized. Here, we co-inoculated HBV and HCV and recapitulated coinfection in immunocompetent ICR mice. Besides successfully reproducing HBV/HCV coinfection and activation of innate immune response to coinfection *in vivo*, we also found that HBV specific humoral and

cell-mediated immune responses were enhanced by the presence of HCV. In line with our findings, a clinical study has recently reported that the IFN-γ expression of the effector T cells in the HBV active/HCV active patients was higher than that of the other groups including HBV inactive/HCV active, HBV active/HCV inactive and HBV inactive/HCV inactive after *ex vivo* restimulation with anti-CD3 (Tseng et al., 2020), which indicates the efficient HCV replication activates the T cells responses during HBV/HCV coinfection.

Therefore, we proposed that besides protecting the host during the initial period of viral infection, HCV-induced innate immune response plays a key role in shaping the host adaptive immune responses (Bertoletti and Ferrari, 2003; Micco et al., 2013). In a previous study, HBV-specific T cell and antibody responses were proved be significantly enhanced after the treatment of poly (I:C)-an immunostimulant, while as knockout of IFN-α/βR, IFN-γ, or CXCR3 in mice impaired poly (I:C)-induced HBV clearance (Wu et al., 2014), which indicated the crucial role of innate immune response in shaping the cellular and humoral responses against HBV. The mechanism of this immunomodulation of innate immunity on HBV specific immune responses is still unclear and might be mediated by HCV-activated cytokines. IP-10, one of the main cytokines induced during HCV infection, was reported to be negatively correlated with HBV DNA load and HBsAg quantification in HBV patients (Wiegand et al., 2015; Wang et al., 2014). IL-21, an important T cell differentiation and stimulation factor, was significant elevated in the acute HCV

infection (Shen et al., 2020), and could reinvigorate the antiviral activity of HBV specific CD8$^+$ T cells in HBV infected patients (Tang et al., 2019). Except the potential cytokines-mediated mechanisms, HCV also might facilitate HBV clearance by other ways. During HCV infection, blood DCs are found enriched in the liver, suggesting the increased migration of DCs to the liver. The *in vitro* studies followingly demonstrated that HCV envelope glycoprotein E2, as well as sera from HCV-infected patients, inhibits the migration of DCs towards CC-chemokine ligand 21 (CCL21), a CCR7 binding chemokine that is important for their homing to lymph nodes (Nattermann et al., 2006). This leads us to an intriguing hypothesis that HCV impairs the ability of DCs to migrate to the draining lymph nodes, causing them to get trapped in the liver, where is the primary site of HBV infection and replication. It might result in the increase of HBV uptake and better antigen presentation and further triggers stronger HBV specific immune response.

The current study provided new evidence on the involvement of host immune responses, especially the adaptive immune responses, in indirect viral interference between HBV and HCV during coinfection. Our immunocompetent coinfection mouse model is not without limitations. The acute HBV infection is the main limitation which only partially mimics the HBV superinfection in HCV pre-infected patients and does not fully capture the characteristics of persistent coinfection and severe liver diseases such as HCC (hepatocellular carcinoma) in patients. A coinfection model susceptible to both HBV and HCV chronic infection with the developed diseases including HCC would be helpful in further elucidating the true properties of the HBV/HCV coinfection in patients. The current immunocompetent coinfection mouse model provides opportunity for treatment-drugs screening and vaccine study.

5. Conclusions

In this study, we comprehensively investigated the viral replication and host immune responses which might mediate the interference between viruses in HBV/HCV coinfected Huh7-NTCP cells and immunocompetent HCV human receptors transgenic ICR mice. We found that presence of HCV significantly inhibited HBV replication *in vitro* and *in vivo* irrespective of the coinfection order, while HBV did not affect HCV replication. Pathological alteration was coincidently reproduced in coinfected mice. In addition to the participation of innate immune response, an involvement of HCV in up-regulating HBV-specific immune responses was described to facilitate HBV clearance. Our systems partially recapitulate HBV/HCV coinfection and unveil the uncharacterized adaptive anti-viral immune responses during coinfection, which renews the knowledge on the nature of indirect viral interaction during HBV/HCV coinfection.

Data availability

All data and materials are available in the main text or the supplementary materials.

Ethics statement

This study was approved by the Institutional Animal Ethical Committee of Wuhan Institute of Virology, Chinese Academy of Sciences and conducted in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals according to the regulations in the People's Republic of China. ICR mice transgenic for HCV human receptors CD81 and occludin (C/O^{Tg}) were created, bred, screened, and maintained as described previously (Chen et al., 2014). All procedures were performed under isoflurane anesthesia. All efforts were made to minimize any suffering and the number of animals used in the study.

Author contributions

Shuhui Liu: conceptualization, data curation, formal analysis, visualization, writing – original draft, writing – review & editing,

investigation, project administration. Kaitao Zhao: data curation, formal analysis. Xi Su: data curation, formal analysis. Xiaoxiao Gao: resources, methodology, software. Yongxuan Yao: resources, methodology. Ranran Kong: methodology. Yun Wang: resources, validation. Chunchen Wu: funding acquisition, validation. Mengji Lu: writing – review & editing. Xinwen Chen: supervision, funding acquisition, writing – review & editing. Rongjuan Pei: supervision, funding acquisition, writing – review & editing.

Conflict of interest

All authors have no competing interests to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.04.001.

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