Hepatitis D virus replication is sensed by MDA5 and induces IFN-β/λ responses in hepatocytes

Graphical abstract

Highlights
- In contrast to HBV, HDV activates the IFN response in hepatocytes.
- MDA5 is the key pattern recognition receptor sensing HDV replication.
- HDV replication is insensitive to the MDA5-mediated self-induced IFN response.
- IFN treatment doesn’t abolish intracellular HDV replication in vitro.

Authors
Zhenfeng Zhang, Christina Filzmayer, Yi Ni, ..., Florian W.R. Vondran, Ralf Bartenschlager, Stephan Urban

Correspondence
Stephan.Urban@med.uni-heidelberg.de (S.Urban)

Lay summary
In contrast to hepatitis B virus, infection with hepatitis D virus induces a strong IFN-β/λ response in innate immune competent cell lines. MDA5 is the key sensor for the recognition of hepatitis D virus replicative intermediates. An IFN-activated state did not prevent hepatitis D virus replication in vitro, indicating that hepatitis D virus is resistant to self-induced innate immune responses and therapeutic IFN treatment.
Hepatitis D virus replication is sensed by MDA5 and induces IFN-β/λ responses in hepatocytes

Zhenfeng Zhang1, Christina Filzmary1, Yi Ni1, Holger Sültsmann2,3,4, Pascal Mutz1,8 Marie-Sophie Hiet1, Florian W.R. Vondran5,6, Ralf Bartenschlager1,7,8, Stephan Urban1,7,*

1Department of Infectious Diseases, Molecular Virology, University Hospital Heidelberg, Heidelberg, Germany; 2Cancer Genome Research Group, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Im Neuenheimer Feld 460, Heidelberg, Germany; 3Translational Lung Research Center (TLRC), German Center for Lung Research (DZL), Heidelberg, Germany; 4German Cancer Consortium (DKTK), Heidelberg, Germany; 5Regenerative Medicine and Experimental Surgery (RemMediES), Department of General, Visceral and Transplant Surgery, Hannover Medical School, Hannover, Germany; 6German Center for Infection Research (DZIF), Partner Site Hannover-Braunschweig, Hannover, Germany; 7German Center for Infection Research (DZIF)-Heidelberg Partner Site, Heidelberg, Germany; 8Division of Virus-Associated Carcinogenesis, German Cancer Research Center (DKFZ), Heidelberg, Germany

Background & Aims: Hepatitis B virus (HBV) and D virus (HDV) co-infections cause the most severe form of viral hepatitis. HDV induces an innate immune response, but it is unknown how the host cell senses HDV and if this defense affects HDV replication. We aim to characterize interferon (IFN) activation by HDV, identify the responsible sensor and evaluate the effect of IFN on HDV replication.

Methods: HDV and HBV susceptible hepatoma cell lines and primary human hepatocytes (PHH) were used for infection studies. Viral markers and cellular gene expression were analyzed at different time points after infection. Pattern recognition receptors (PRRs) required for HDV-mediated IFN activation and the impact on HDV replication were studied using stable knock-down or overexpression of the PRRs.

Results: Microarray analysis revealed that HDV but not HBV infection activated a broad range of interferon stimulated genes (ISGs) in HepG2NTCP cells. HDV strongly activated IFN-β and IFN-λ in cell lines and PHH. HDV induced IFN levels remained unaltered upon RIG-I (DDX58) or TLR3 knock-down, but were almost completely abolished upon MDA5 (IFIH1) depletion. Conversely, overexpression of MDA5 but not RIG-I and TLR3 in HuH7.5NTCP cells partially restored ISG induction. During long-term infection, IFN levels gradually diminished in both HepG2NTCP and HepaRCNTCP cells. MDA5 depletion had little effect on HDV replication despite dampening HDV-induced IFN response. Moreover, treatment with type I or type III IFNs did not abrogate HDV replication.

Conclusion: Active replication of HDV induces an IFN-β/λ response, which is predominantly mediated by MDA5. This IFN response and exogenous IFN treatment have only a moderate effect on HDV replication in vitro indicating the adaption of HDV replication to an IFN-activated state.

Keywords: Hepatitis D virus; Hepatitis B virus; Innate immunity; Pattern recognition receptor; MDA5; Interferon induction; Interferon treatment; Hepatocytes.

Introduction

Among the 240 million hepatitis B virus (HBV)-infected people worldwide,1 15–25 million are coinfected with hepatitis D virus (HDV) (WHO fact sheet, July 2016), a satellite virus which requires HBV envelope proteins for particle assembly and spread. HBV/HDV co-infection leads to the most severe form of viral hepatitis with an accelerated progression to liver fibrosis, cirrhosis and hepatocellular carcinoma.2 Therapeutic options for chronically HBV/HDV coinfected patients are still limited to IFN-α therapy for eligible patients, however, this therapy is not curative in the vast majority of patients.3,4 HDV consists of a single-stranded circular 1.7 kb RNA genome of negative polarity and represents the smallest mammalian virus genome identified to date.5 HDV RNA replication takes place in the nucleus of hepatocytes through a double rolling circle mechanism, producing antigenomic RNA, genomic RNA and mRNAs encoding the small and large hepatitis delta antigens (S- and L-HDAg). The circular genomic HDV RNA is highly self-complementary (~74% base pairing) and therefore forms rod-shaped viroid-like tertiary structures.6 During assembly, the newly synthesized genomic RNA associates with HDAg molecules to form a ribonucleoprotein (RNP) complex.7 These RNPs become enveloped by budding into the ER lumen at HBV envelope protein (L-/M- and S-HBsAg) assembly sites, which are provided by either covalently closed circular DNA of HBV coinfected hepatocytes or possibly integrated HBV sequences.8

The cellular innate immune system constitutes the first line of defense against microbial infections and plays a crucial role in
Results

**HDV infection activates the IFN system in HepG2NTCP and HepaRG cells**

To investigate the cellular response towards HDV and HBV infection, in depth, we performed whole transcriptome analysis of infected, innate immune competent HepG2NTCP cells. Myrcludex B served as entry inhibitor control to rule out effects mediated by contaminants in the virus inoculum. Further, we used HuH7NTCP cells, which support HDV infection but are deficient in IFN production. 

Infection efficiency was verified by immunofluorescence (IF) staining of HDAg or HBcAg (Fig. 1A), and total RNA was subjected to microarray analysis at day nine post infection. Compared to uninfected and MyrB-inhibited HepG2NTCP cells, 426 genes were upregulated (>2-fold) by HDV (Fig. 1B, left column). Remarkably, HBV infection of the same cell line did not result in any significant (>2-fold) induction of gene expression (Fig. 1B, right column). Pathway clustering analysis revealed that the IFN signaling pathway was the most significant upregulated pathway (Fig. S1) with the 15 most upregulated (>10-fold) genes being ISGs (Fig. 1C). Notably, MyrB treatment blocked ISG activation (Fig. 1C), indicating that productive HDV infection rather than the inoculum itself is required. Results from the microarray assay were validated for selected ISGs including IFI27, IFI44, ISG15, MX1, and RSAD2 by RT-qPCR (Fig. 1D).

To determine the kinetics of HDV-mediated activation of the IFN system in a more authentic cell culture system, differentiated HepaRG cells expressing only endogenous NTCP were used. After differentiation, cells were inoculated with HDV in the absence or presence of MyrB. HDAg and MX1 expression were analyzed by IF at the indicated time points. As depicted in Fig. 1E (upper panels), HDAg (red) could be detected from day one post infection onwards. MX1 expression (green) was detectable already on day three post infection and rapidly spread throughout the cell culture. Both HDAg expression and MX1 induction could be inhibited by MyrB indicating that MX1-induction requires HDV replication and is not caused by the inoculum per se.

**HDV activates IFN-ß and IFN-λ1 in HepG2NTCP, HepaRGNTCP cells and PHH**

To identify the pattern of IFNs induced upon HDV infection, HepG2NTCP, HepaRNCTP cells and PHH were infected with HDV at 0.5 IU/cell in the absence and presence of MyrB. Infection efficacy and successful block of virus entry was controlled by IF of HDAg at day five post infection (Fig. 2A, left panels). Intracellular HDV RNA replication was quantified by RT-qPCR at day one, three, five and seven post infection (Fig. 2A, right panels). Replicative intermediates were detectable in all cell lines from day three on (Fig. 2A, right panels). Further, mRNAs of IFNα, -ß, -λ1 and -λ2/3 at the same time points were quantified by RT-qPCR. As shown in Fig. 2B, activation of IFNβ and IFNλs, but not IFNα was measurable from day three on, peaking at day five to seven. MyrB completely blocked IFN activation.

Materials and methods

**Cells**

HBV or HDV vectors of human sodium-taurocholate co-transporter polypeptide (NTCP [SLC10A1]) overexpressing cell lines (HepG2NTCP, HuH7NTCP and HepaRGNTCP) and HCV luciferase replicon cell lines (Luc-ubi-neo/ET) were generated in our previous studies. 

Huh7.5 cells were kindly provided by C.M. Rice, Rockefeller University, USA. ShRNA-mediated knock-down in HepG2NTCP and HepaRNCTP cell lines and over-expression of RIG-I, MDA5 and TLR3 in Huh7.5NTCP cells were generated by lentiviral transduction in this study as described. 

All of the cell lines used in this study were authenticated and free of mycoplasma. PHH were isolated from liver resections of patients undergoing partial hepatectomy as described. All tissue donors gave written informed consent for the experimental use of their liver specimen.

**Plasmids and viruses**

NTCP vectors and RIG-I, MDA5 and TLR3 encoding lentiviral pWPI vectors have been described before. For generation of RIG-I, MDA5 and TLR3 knock-down constructs, shRNAs were produced by PCR using Fw-XhoI-5′ (5′-aagggctagcaaggtatactgtt gcagctgag-3′) and Rev-EcoRI-3′ (5′-agccccttgaattccgaggcag taggca-3′) as primers and oligonucleotides listed in Table S1 as the template. PCR fragments were inserted into the XhoI/ EcoRI sites of the lentiviral pPAM vector. The HDV antigenomic RNA encoding plasmid pJC126 (genotype 1) was kindly provided by Dr. John Taylor (Fox Chase Cancer Center, USA). The HBV envelope protein encoding plT7HB2.7 vector (genotype D) was a gift from Camille Sureau (INTS, France). HBV was produced in HepAD38 cells. HBV was obtained by co-transfection of HuH7 cells with plasmids pJC126 and pT7HB2.7. Viruses were purified by heparin affinity chromatography.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

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**Viral Hepatitis**

inhibition and clearance of invading pathogens. The innate immune response is also crucial for mounting robust adaptive immune response (reviewed in) and thereby determine the outcome of infection, i.e. clearance or persistence, with the latter often associated with chronic inflammation. Innate immune responses are initiated by the recognition of pathogen-associated molecular patterns (PAMPs), such as viral genomes or replication intermediates like double-stranded RNA (dsRNA). Thereby, pattern recognition receptors (PRRs) i.e. toll-like receptor 3 (TLR3), the retinoic acid inducible gene I (RIG-I [DDX58])–like receptors (RLRs) including RIG-I and the melanoma differentiation antigen 5 (MDA5 [IFIH1]) (reviewed in) sense the PAMPs and initiate a cascade of signaling events that lead to the production of type I (IFN-α/β) and type III interferons (IFN-λ). These are secreted and bind their cognate IFN-receptors on the membrane of infected and non-infected neighboring cells, activating hundreds of interferon stimulated genes (ISGs) that counteract viral infections by diverse mechanisms.

Preceding studies suggest that HDV induces IFN-β and IFN-λ, in differentiated HepaRG cells and various mouse models. However, it remains unclear: i) which PRR(s) are sensing HDV, ii) which ISGs are specifically induced and iii) how a possible induction of ISGs and the resulting antiviral state of cells influences HDV replication. Herein, we took advantage of different in vitro infection systems to identify the PRR sensing HDV and the types of ISGs induced by the virus. Moreover, we determined the impact of the IFN-induced antiviral state on HDV replication.
with HDV or HBV and HuH7 NTCP cells with HDV in the absence or presence of the entry inhibitor MyrB. (A) At day nine post infection, infection efficiency was analyzed by IF detecting HDAg and HBcAg. (B) Cellular transcripts were quantified by microarray. Numbers of up- or downregulated genes (fold change >2) from HDV infected HepG2 NTCP and HepaRG NTCP cells significantly inhibited HCV replication. (Fig. S2). This indicates that IFN-β and –λ, activation is triggered neither by incoming viral RNP complexes nor by HDAgs, but requires active replication of HDV RNA.

To test whether HDV-induced IFNs show antiviral activity, we applied the supernatants of HDV-infected HepG2NTCP and HepaRGNTCP cells to an HCV luciferase replicon-based reporter system. As shown (Fig. 2C), cell culture supernatants (day 5–7) from HDV infected HepG2NTCP and HepaRGNTCP cells significantly inhibited HCV replication.

**MDA5 is the key PRR sensing HDV**

To determine PRR(s) possibly involved in HDV sensing we first evaluated the functionality of the key sensors RIG-I, MDA5 and TLR3 in HepG2NTCP- and HepaRGNTCP cell lines by treatment with PRR-specific stimuli: SeV (stimulation of RIG-I), a mutant of the Mengo-Zn virus 29 (stimulation of MDA5) or incubated the cells with poly(I:C) (activation of TLR3). Quantification of the induced IFNs showed that both HepG2NTCP and HepaRGNTCP cell lines are competent in RIG-I and MDA5 activation, while only HepaRGNTCP cells are competent in TLR3 signaling (Fig. 3A).

We next generated HepG2NTCP and HepaRGNTCP cell lines stably expressing RIG-I, MDA5 or TLR3-specific shRNAs resulting in the knock-down of the respective PRR. Quantification of the mRNAs in both cell lines demonstrated efficient suppression of each receptor (Fig. 3A, left panels). For RIG-I and MDA5, this could be verified at the protein level (Fig. 3A, right panels), while TLR3 could not be detected in western blot. Knock-down was further confirmed by using a functional assay demonstrating a loss of IFN-β induction by SeV and Mengo-Zn virus infection as well as poly(I:C) treatment (Fig. 3B). Taking advantage of these cell lines, we quantified HDV replication and activation of the IFN response upon HDV infection. As depicted (Fig. 3B and C), infection efficacy was comparable in all six PRR knock-down cell lines, as shown by HDAg-specific
Fig. 2. HDV infection induces IFN-β and IFN-λ in NTCP-expressing cell lines and PHH. HepG2<sup>NTCP</sup> cells, HepaRG<sup>NTCP</sup> cells and PHH were infected with HDV in the absence or presence of MyrB. (A) Infection efficiency of HDV was analyzed by IF against HDAg at d5 p.i. (left panels, scale bars: 100 µm). Copy numbers of (A) intracellular HDV RNA (right panels) and (B) IFN-α, -β, -κ1 and -κ2/3 mRNAs at day 1, 3, 5 and 7 p.i. were quantified using RT-qPCR. (C) Inhibitory activity of serial diluted SN from HDV infected HepG2<sup>NTCP</sup> and HepaRG<sup>NTCP</sup> cells (day 5–7 p.i.) on HCV replication. Experiment setting is shown in left panel. Relative luciferase activity was measured and normalized to cells treated with mock infected SN (mock) (right panels). Values in IF images depict the mean percentage of HDAg positive cells (n = 4). Values of qPCR and relative luciferase activity are shown as mean ± SD (n = 3). HCV, hepatitis C virus; HDAg, hepatitis D antigen; HDV, hepatitis D virus; IF, immunofluorescence; IFN, interferon; LOD, limit of detection; MyrB, Myrcludex B; PHH, primary human hepatocyte; p.i., post infection; qPCR, quantitative PCR; SN, supernatants.
Fig. 3. shRNA-mediated knock-down of MDA5 impairs HDV-induced IFN response in HepG2NTCP and HepaRGNTCP cells. (A) Generation of HepG2NTCP- and HepaRGNTCP-derived cell lines with stable knock-down of RIG-I, MDA5, TLR3 or expressing a non-targeting control shRNA (NT). Knock-down efficiency was analyzed at mRNA level using RT-qPCR (upper panels) and at protein level using western blot (lower panel) (ns, non-specific). Note that TLR3 protein was not detected. (B) HepG2NTCP- and (C) HepaRGNTCP-derived knock-down cell lines were infected with HDV. At day five post infection, infection efficiency was analyzed by IF detecting HDAg (scale bars: 200 μm). (B and C, upper panels) Copy numbers of intracellular HDV RNA and (D) of the mRNAs for IFNβ, IFNλ1 and the ISG RSAD2 (see Fig. 1C) were quantified by RT-qPCR. Values in IF images depict the mean percentage of HDAg positive cells (n = 4). Values of qPCR are shown as mean ± SD (n = 3). HDAg, hepatitis D antigen; HDV, hepatitis D virus; IF, interferon; ISG, IFN-stimulated gene; RT-qPCR, reverse transcription quantitative PCR; shRNA, short hairpin RNA.
IF performed at day five post infection, and resembled that of the control cell lines expressing a non-targeting shRNA (right panels). Consistently, intracellular HDV RNA replication was comparable between all different cell lines (Fig. 3B and C, left panels). Notably, activation of IFN-β, -α1 and the ISG RSAD2 was almost completely blocked in the MDA5-deficient cell lines whereas knock-down of RIG-I and TLR3 had no effect on IFN activation (Fig. 3D). Similar blockade of IFN response by depletion of MDA5, but not RIG-I and TLR3, was observed using alternative shRNAs for each PRR (Fig. S4) ruling out possible off-target effects. This provides compelling evidence that MDA5 is the key PRR for HDV in HepG2NTCP and HepaRGNTCP cells.

**Overexpression of MDA5 promotes HDV-induced IFN response in HuH7.5NTCP cells**

HuH7.5 cells are deficient in sensing through RLRs and TLRs. However, HuH7.5 cells can promote RLR signaling upon ectopic expression of these PRRs making these cells a preferred tool to investigate RLR-mediated responses. Consistently, we observed a very weak induction of IFN-λ1 in HuH7.5NTCP cells upon infection with SeV or Mengo-Zn virus or after treatment with poly(I:C) (Fig. 4B). Yet, stable expression of RIG-I, MDA5 or TLR3 (Fig. 4A) resulted in a profound enhancement of IFN-λ induction by SeV and Mengo-Zn virus infection (Fig. 4B). Though, poly(I:C) mediated stimulation of the TLR3 pathway still did not induce an IFN response, arguing for an additional downstream defect (Fig. 4B).

We finally infected HuH7.5NTCP cells stably expressing RIG-I, MDA5 or TLR3 with HDV. IF staining of HDAg and quantification of total intracellular HDV RNA revealed comparable infection efficiencies in all cell lines (Fig. 4C and D). Although the HDV-induced IFN response was generally low, we observed a significant activation of RSAD2 and IFI44 upon HDV infection in the MDA5 overexpressing cells, but not in cells overexpressing RIG-I (Fig. 4E). This provides additional evidence for MDA5 being the sensor of HDV RNA.

**IFN activation during long-term HDV infection of HepG2NTCP and HepaRGNTCP cells**

HDV coinfection in chronic HBV-infected patients typically manifests as a persistent infection, depending on enduring replication and intrahepatic spread of HDV replicative RNA intermediates. To find out if the induced IFN response interferes with enduring HDV replication or vice versa, we performed long-term cell culture infection experiments in HepG2NTCP and HepaRGNTCP cells. We infected both cell lines with HDV and analyzed HDag expression by IF (Fig. 5A), L- and S-HDag by western blot (Fig. 5B) and intracellular HDV RNA by RT-qPCR (Fig. 5C) throughout a 23 day infection period. HDag expression and HDV RNA replication in HepG2NTCP cells peaked at day 11 post infection and remained at such levels until day 23 (Fig. 5A, B and C, upper panels). In contrast, HDag expression and HDV RNA replication in the more authentic HepaRG NTCP cell line dropped profoundly after day five post infection and were almost undetectable at day 23 (Fig. 5A, B and C, lower panels). These data indicate a marked difference in the persistence of HDV RNA replication in HepG2NTCP vs. the HepaRGNTCP cell line. In parallel, we quantified activation of IFN-α, -β, -α1, -α2/3 and...

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**Fig. 4. Overexpression of MDA5 enhances HDV-induced IFN response in HuH7.5NTCP cells.** (A) Quantification of RIG-I, MDA5 and TLR3 mRNA in HuH7.5NTCP cells and cell lines derived thereof stably expressing indicated PRRs by RT-qPCR. (B) Cells were infected with SeV, or Mengo-Zn virus, or treated with poly(I:C) added into the medium. After 24 h, copy numbers of IFN-λ1 mRNA were quantified using RT-qPCR and fold changes were computed. (C-E) HuH7.5NTCP and cell lines derived thereof were infected with HDV. (C) Infection efficiency was determined by IF detecting HDag (scale bars: 100 μm). Copy numbers of (D) intracellular HDV RNA and (E) mRNAs of the ISGs RSAD2 and IFI44 were quantified by RT-qPCR. Values in IF images depict the mean percentage of HDag positive cells (n = 4). Values of qPCR are shown as mean ± SD (n = 3). *p < 0.05 by Student’s t test. HDAg, hepatitis D antigen; HDV, hepatitis D virus; IF, interferon; RT-qPCR, reverse transcription quantitative PCR.
RSAD2. Consistent with the results depicted in Fig. 2B, HepG2NTCP cells (Fig. 5D, upper row) and HepaRGNTCP cells (Fig. 5D, lower row) showed a strong increase of all IFN and ISG mRNAs except IFNα at five days post infection. However, at later points IFN and ISG mRNA levels decreased significantly in both cell lines (Fig. 5D). Despite this drop, the intracellular HDV RNA level remained unchanged until day 23 in HepG2NTCP cells, while it significantly dropped in HepaRGNTCP cells (Fig. 5C).

Again, MyrB abrogated all signals, verifying the requirement for active replication. This result suggests that although HDV RNA triggers an IFN response, this response declines at later time points of infection.

Effect of MDA5 depletion on HDV replication

To investigate whether the MDA5-mediated, HDV-induced IFN-response inhibits HDV replication during long-term cell culture infection, we determined HDV replication markers (HDV RNA and HDAg) in HepG2NTCP and HepaRGNTCP cell lines harboring a stable knock-down of MDA5 (shMDA5) and compared them to the parental cells expressing a non-targeting control shRNA (shNT). HDAg expression (Fig. 6A and B) and intracellular HDV RNA was quantified until day 23 post infection (Fig. 6C). We found that HDAg expression and HDV RNA increased from day 1–11 post infection and remained at high levels until day 23 in both HepG2NTCP derived cell lines. In the two HepaRGNTCP cell lines, HDAg expression and HDV RNA decreased significantly after day seven post infection. These data confirm previous result in HepG2NTCP and HepaRGNTCP cells (Fig. 5). Importantly, although HDV RNA levels in HepG2NTCPshMDA5 and HepaRGNTCPshMDA5 cells were higher than in the control HepG2NTCPshNT and HepaRGNTCPshNT cells, the level of enhancement was rather weak at early points and getting moderate at late points of infection (about 7.5-fold difference between HepaRGNTCPshNT and shMDA5) (Fig. 6C). The kinetics of IFN response (IFNα, IFNβ and RSAD2 mRNA levels) correlated with that of HDV RNA, and the overall IFN response in shMDA5 cells was significantly lower than that in shNT cells (Fig. S5). These results imply that HDV replication is only moderately sensitive to MDA5-mediated innate immune responses, however, the MDA5 mediated antiviral IFN response may contribute to the reduction of HDV markers during long-term cell culture infection.

HDV replication is insensitive to IFN responses

As HDV replication is only moderately affected by the IFNs induced during replication, we analyzed if ISG expression was dampened in HDV positive cells on the single cell level. HDV...
Fig. 6. Effect of MDA5 knock-down on HDV replication in HepG2NTCP and HepaRGNTCP cells. HepG2NTCP- and HepaRGNTCP-derived cell lines expressing shRNA against MDA5 or a non-targeting shRNA control (NT) were infected with HDV and harvested at day 1, 3, 5, 7, 11, 17 or 23 post infection. (A) At each time point HDAg expression was determined by IF (scale bar: 100 μm) and (B) western blot. (C) Copy numbers of intracellular HDV RNA were quantified by RT-qPCR. Values in IF images depict the mean percentage of HDAg positive cells (n = 4). Values of qPCR are shown as mean ± SD (n = 3). * p <0.05; ** p <0.01; *** p <0.001 by Student’s t test. HDAg, hepatitis D antigen; HDV, hepatitis D virus; IF, immunofluorescence; LOD, limit of detection; RT-qPCR, reverse transcription quantitative PCR; shRNA, short hairpin RNA.

infected HepG2NTCP- and HepaRGNTCP cells showed high levels of Mx1, comparable to HDAg negative cells (Fig. 7A). This indicates that, at the single cell level, HDV replication is insensitive to IFN responses without blocking ISG expression.

To test whether HDV replication is sensitive to exogenous IFN treatment, HepG2NTCP and HepaRGNTCP cells were infected with HDV, and treated with IFN-α2a (100 IU/ml) or IFN-λ1 (10 ng/ml) from day 1–7 (early treatment) or day 5–11 (late treatment) (Fig. 7B). HDAg and HDV RNA were analyzed after IFN treatment. Both IFN-α2a and IFN-λ1 treatments showed less than 50% inhibition of HDV replication markers during early treatment (day 1–7) and no significant effect during late treatment (day 5–11) in both HepG2NTCP and HepaRGNTCP cell lines (Fig. 7C and D), although both IFN treatments severely inhibited HCV replicon replication (Fig. 7E). This result indicates that HDV replication is insensitive to exogenous IFN treatment after the establishment of infection in vitro.

Discussion

Our study shows that (i) HDV replication, in contrast to HBV replication, activates IFN-β and IFN-λ in NTCP-expressing HepG2NTCP and HepaRGNTCP cell lines and in PHH, (ii) MDA5 is the key sensor for IFN activation by recognizing replicating HDV RNA intermediates, (iii) knock-down of MDA5 has little effect on HDV replication efficacy, and (iv) HDV replication is not abolished by the endogenously induced IFN response or exogenous IFN treatment.

Type I (IFN-α/β) and type III IFNs (IFN-λ) are potent antiviral components of the cellular innate immune system. Although both IFNs activate a similar spectrum of ISGs, they exert distinct and non-redundant functions. Previous results have shown that IFN-β, but not IFN-α is activated by HDV infection. Further, a moderate induction of IFN-λ was observed in HDV/HBV coinfected humanized mice. Consistent with these reports, we found that HDV infection activates IFN-β and IFN-λ1/2/3 but not IFN-α (Fig. 2) in NTCP-expressing cell lines and PHH. IFN-induction by HDV requires active RNA replication since UV irradiated, inactivated hepatitis D virions and ectopically expressed HDAg had no effect on IFN expression (Fig. S2).

Through shRNA-mediated knock-down of the three key RNA PRRs RIG-I, MDA5 and TLR3, we identified MDA5 as the key sensor recognizing HDV in HepG2NTCP and HepaRGNTCP cells (Fig. 3). MDA5 belongs to the group of RLRs and senses predominantly long dsRNA or higher ordered RNA structures located in the cytosol. However, since HDV replicates in the nucleus forming antigenomic and genomic multimeric and monomeric RNAs intermediates, these are probably not accessible to MDA5. Thus, we assume that RNA sensing by MDA5 might occur during cytosolic export of RNPs during cytoplasmic trafficking to the
site of envelopment. Because of their self-complementarity, the rod-shaped secondary structure elements within the HDV genome may be important for efficient recognition by MDA5. However, a detailed investigation of the crucial RNA elements recognized by MDA5 activation remains for future analysis and will allow a better molecular understanding of both MDA5 activation and HDV induced IFN response.

After HDV superinfection of HBV mono-infected patients, HDV regularly persists in a chronic state. Thus, in the majority of cases IFN-induction does not lead to elimination of HBV nor HDV (although HBV replication is often suppressed in HDV/HBV coinfected patients). We therefore analyzed the HDV replication kinetics and innate immune responses during long-term cell culture infection (up to 23 days) of HepG2 NTCP and HepaRG NTCP cells. HDV RNA replication kinetics are similar at early time points (day 1–7) in both cell lines, and stay at a peak plateau in HepG2 NTCP cells but decline profoundly in the more authentic HepaRG NTCP cells (Fig. 5) at later time points. Of note, the innate immune responses significantly decreased from day seven post infection in both cell lines (Fig. 5).

**Fig. 7.** HDV replication is insensitive to IFNs treatment. (A) HepG2 NTCP and HepaRG NTCP cells were infected with HDV. At day five post infection, HDV replication and Mx1 activation at single cell level were analyzed by IF (scale bars: 50 μm). Arrows indicate HDAg and Mx1 co-positive cells. (B-D) HDV infected HepG2 NTCP and HepaRG NTCP cells were treated with (B) IFN-α2α (100 IU/ml) or IFN-λ1 (10 ng/ml) from day 1–7 (d1-7) or day 5–11 (d5-11). (C) HDAg expression was analyzed by IF (scale bar: 200 μm), and (D) copy numbers of intracellular HDV RNA were quantified by RT-qPCR. Inhibitory activity of serial diluted IFN-α2α (undiluted: 100 IU/ml) and IFN-λ1 (undiluted: 10 ng/ml) on HDV replication (see Fig. 2C for experiment setting). Relative luciferase activity was measured and normalized to mock treated cells. Values in IF images depict the mean percentage of HDAg positive cells (n = 6). Values of qPCR and relative luciferase activity are shown as mean ± SD (n = 3). HCV, hepatitis C virus; HDAg, hepatitis D antigen; HDV, hepatitis D virus; IF, immunofluorescence; IFN, interferon; RT-qPCR, reverse transcription quantitative PCR.
HDV RNA replication from day five post infection in HepaRG<sub>NTCP</sub> cells is probably not caused by the inhibition of the innate immune responses, since MD5 depletion in the same cell line has little effect on HDV replication (Fig. 6). The decline of IFN activation in the presence of continuously high level replication of HDV RNA in HepG2<sub>NTCP</sub> cells may thus be due to antagonists produced by HDV or the negative feedback of IFN signaling pathway as it has been reviewed in.<sup>[10]</sup> Previous in vitro studies using transfection of HDV encoding plasmid reported an HDV-mediated inhibition of STAT1 nuclear translocation in response to IFN-α in HuH7 cells.<sup>[40]</sup> Using the humanized mouse model Giersch et al. observed that STAT1 nuclear accumulation was most pronounced in human hepatocytes displaying undetectable amounts of HDAg.<sup>[17]</sup> Conversely, our results show that activation of IFN-induced Mx1 protein was not reduced in HDV replicating HepG2<sub>NTCP</sub> and HepaRG<sub>NTCP</sub> cells (Fig. 7A). This indicates that IFN signaling still occurs in HDV infected cells and favors other mechanisms that contribute to the reduction of innate immune responses at late time points.

Depletion of MD5 had a weak effect on HDV replication, but may contribute to HDV inhibition during long-term cell culture infection (Fig. 6). However, since the replication of HCV is still strongly inhibited by supernatants from HDV infected HepG2<sub>NTCP</sub> and HepaRG<sub>NTCP</sub> cells (Fig. 2C), we conclude that HDV replication is essentially insensitive to MD5-mediated IFN response.

We confirmed this assumption by analyzing the sensitivity of HDV replication to ectopically added IFN-α and IFN-λ. Although both IFNs strongly inhibited HCV replication (at 100 U/ml) (Fig. 7E), they exhibited only a moderate effect (<50% inhibition) on HDV replication. Moreover, this inhibition was only observed when we treated the cells at an early stage of infection (day 1–7) but no effect after the establishment of infection (day 5–11) (Fig. 7C and D), indicating the insensitivity of HDV replication to intracellular establishment of infection. One previous study also showed that the inhibition of HDV replication by IFN-α and IFN-γ in PHH only occurred when the cells were exposed to these IFNs at a very early stage (<16 h) of infection.<sup>[34]</sup> Of note, our study was performed using HDV mono-infection in in vitro cell culture models that do not support secondary infections (due to the absence of HBV envelope proteins). During HBV/HDV coinfection in patients or spread-supporting models, innate immune responses may influence HDV spread. Recently reported suppression of HDV replication by pegylated (Peg) IFN-α and IFN-λ in HBV/HDV coinfected humanized mice<sup>[42]</sup> and clinical observations of HDV inhibition by IFNs in some patients might be due to an inhibition of viral spread rather than to a direct effect on replication in persistently infected hepatocytes. These findings may also explain the clinical observations of low response rate following IFN therapy and HDV RNA relapse in chronic HDV infected patients (reviewed in<sup>[43]</sup>).

Taken together, we demonstrate that HDV activates IFN-β and IFN-λ responses, through activation of MD5 by unknown replicative intermediate structure(s) of HDV RNA. Intracellular HDV replication is mostly insensitive to the MD5 mediated innate immune response and, similar to HBV,<sup>[44]</sup> not directly affected by exogenous IFNs after the establishment of infection. These findings contribute to a better understanding of the interaction between HDV and the innate immune system, helping to explain the limited efficacy of current IFN therapies.

### Financial support
This work received funding by German Center for Infectious Research (DZIF), TTU Hepatitis, Project 5.807 and 5.704, the Deutsche Forschungsgemeinschaft (DFG) SFB1129 (TP16) and TRR179 (TP15, TP9).

### Conflict of interest
Prof. Dr. Stephan Urban holds patents on the use of Myrcludex B. Other authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

### Authors' contributions

### Acknowledgement
We thank the microarray unit of the DKFZ Genomics and Proteomics Core Facility for providing the Illumina Whole-Genome Expression Beadchips and related services. We are grateful to Florian A. Lempp, Bingqian Qu, Nadine Gillich, Silke Bender, Oliver Grünvogel, and Christopher Dächert for providing materials and help for the experiments. We thank Franziska Schlund and Anja Rippert for lab organization and cell culture support. We also thank Drs. Volker Lohmann, Alessia Ruggieri, Macro Binder and other members in the department of infectious diseases in University Hospital Heidelberg for useful discussions.

### Supplementary data
Supplementary data associated with this article can be found, in the online version, at [https://doi.org/10.1016/j.jhep.2018.02.021](https://doi.org/10.1016/j.jhep.2018.02.021).

### References
Author names in bold designate shared co-first authorship


