



# Inducers of the NF- $\kappa$ B pathways impair hepatitis delta virus replication and strongly decrease progeny infectivity *in vitro*

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**Background & Aims:** HDV superinfection of chronically HBV-infected patients is the most aggressive form of chronic viral hepatitis, with an accelerated progression towards fibrosis/cirrhosis and increased risk of liver failure, hepatocellular carcinoma, and death. While HDV infection is not susceptible to available direct anti-HBV drugs, suboptimal responses are obtained with interferon- $\alpha$ -based therapies, and the number of investigational drugs remains limited. We therefore analyzed the effect of several innate immune stimulators on HDV replication in infected hepatocytes.

**Methods:** We used *in vitro* models of HDV and HBV infection based on primary human hepatocytes (PHHs) and the non-transformed HepaRG cell line that are relevant to explore new innate immune therapies.

**Results:** We describe here, for the first time, anti-HDV effects of Pam3CSK4 and BS1, agonists of Toll-like receptor (TLR)-1/2, and the lymphotoxin- $\beta$  receptor (LT $\beta$ R), respectively. Both types of agonists induced dose-dependent reductions of total intracellular HDV genome and antigenome RNA and of HDV protein levels, without toxicity in cells monoinfected with HDV or co/superinfected with HBV. Moreover, both molecules negatively affected HDV progeny release and strongly decreased their specific infectivity. The latter effect is particularly important since HDV is thought to persist in humans through constant propagation.

**Conclusions:** Immune-modulators inducing NF- $\kappa$ B pathways in hepatocytes can inhibit HDV replication and should be further evaluated as a possible therapeutic approach in chronically HBV/HDV-infected patients.

**Lay summary:** Hepatitis delta virus causes the most severe form of viral hepatitis. Despite positive recent developments, effective treatments remain a major clinical need. Herein, we show that immune-modulators that trigger the NF- $\kappa$ B pathways could be effective for the treatment of hepatitis delta infections.

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## Introduction

HDV/HBV coinfection is the most aggressive chronic form of viral hepatitis<sup>1,2</sup> with an accelerated progression towards fibrosis/cirrhosis followed by clinical liver decompensation and related death.<sup>3</sup> HDV virions contain a ribonucleoprotein (RNP) composed of a circular single-stranded negative RNA genome presenting a “quasi” double-stranded conformation and viral

proteins called HD antigens (HDAGs). HDV is a defective satellite virus that uses HBV envelope proteins to egress from and re-enter into hepatocytes. Apart from those steps, HDV intracellular RNA replication is known to be independent of HBV but is highly dependent on the cellular machinery (see<sup>4</sup> for review). Briefly, in the nucleus of infected cells, incoming HDV genomes (HDV-Gs) serve as a template for the synthesis of replicative intermediates called anti-genomes (HDV-AG), which are fully complementary to the HDV genome sequence and bear the open reading frame for HDAG protein synthesis. The small HDAG (S-HDAG) is produced early after infection by transcription of HDV mRNAs from the incoming HDV-G within the HDV RNP and large HDAG (L-HDAG; that displays a C-terminal extension of 19 amino acids compared to S-HDAG) is produced later after editing of the amber termination codon on HDV-AG.

No direct-acting agents, *i.e.* molecules specifically targeting viral enzymatic activities, against HDV are currently approved to treat chronic HDV infection because – apart from viral ribozyme

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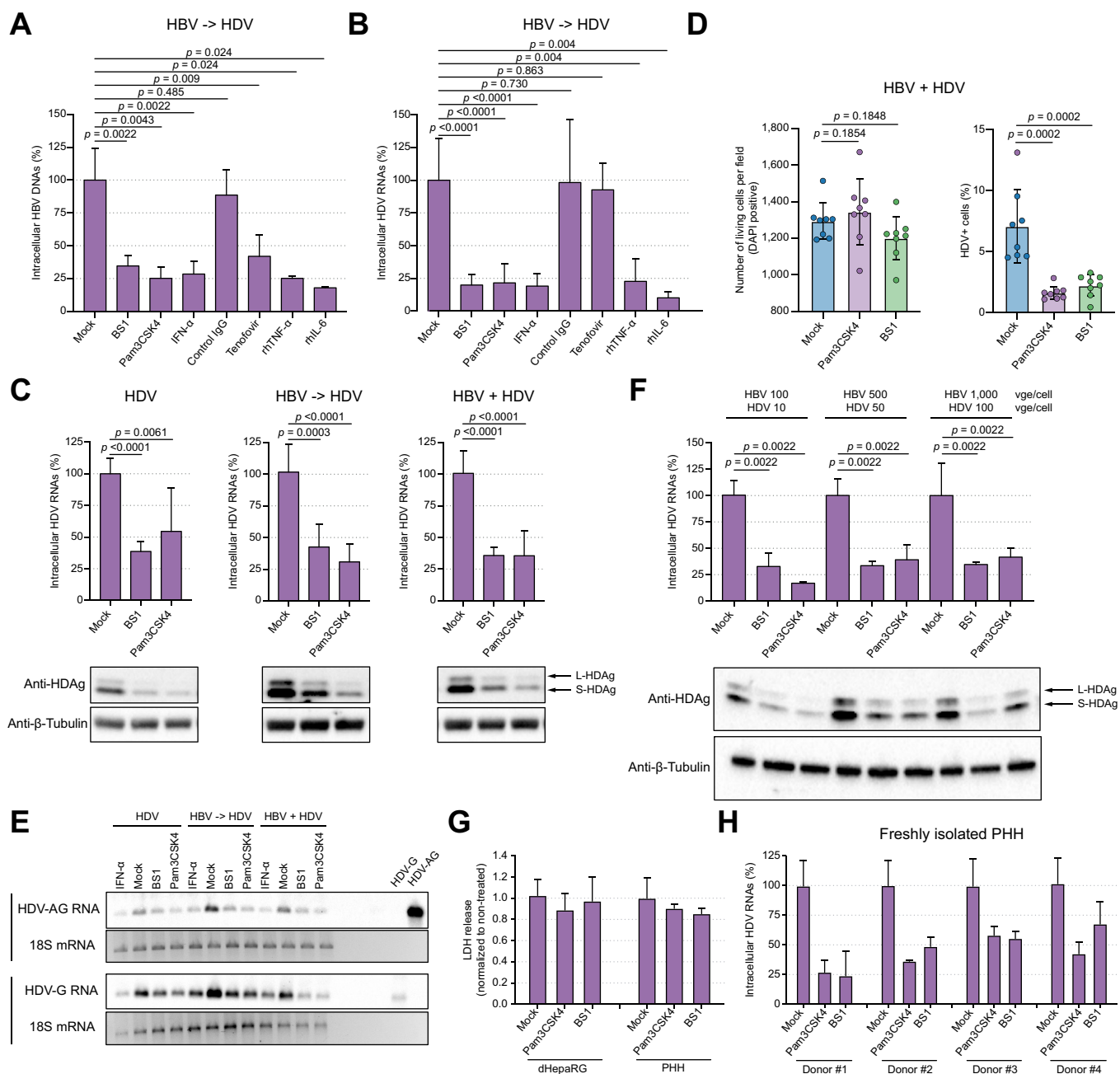
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**Fig. 1. Inducers of the NF-κB pathway such as Pam3CSK4 and BS1 reduced the levels of intracellular HDV RNA and protein.** (A, B) dHepaRG cells were infected with HBV and then with HDV 6 days later with 100 vge/ml per cell for HBV and 10 vge/ml per cell for HDV. Cells were treated 3 days after HDV infection with BS1 (0.5 μg/ml) or Pam3CSK4 (0.5 μg/ml), IFN-α (1,000 U/ml), control IgG (0.5 μg/ml), tenofovir (10 μM), TNF-α (50 ng/ml) or recombinant human IL-6 (rhIL-6, 100 ng/ml) for 11 days. Cells were collected and the levels of total intracellular (A) HBV DNAs and (B) HDV RNAs were analyzed by qPCR and RT-qPCR respectively. Results are the mean ± SD of 2 (for HBV RNAs) or 3 (for HDV RNAs) independent experiments each performed with 3 biological replicates and statistical analyses were performed using Mann-Whitney tests. (C,D,E) dHepaRG cells were infected with HDV (HDV), HBV and with HDV 6 days later (HBV->HDV) or coinfecting with HBV and HDV (HBV+HDV) with 100 vge per cell for HBV and 10 vge per cell for HDV. Cells were treated 3 days after HDV infection with (C, D) BS1 (0.1 μg/ml), Pam3CSK4 (0.1 μg/ml) or (E) IFN-α (1,000 U/ml) for 11 days. Cells were collected and the levels of total (C, E) intracellular HDV RNAs as well as the levels (C) HDV proteins were analyzed by (C) RT-qPCR, (E) Northern Blot or (C) western blot, respectively. (C) Results are the mean ± SD of 2 independent experiments each performed with 4 biological replicates and statistical analyses were performed using Mann-Whitney tests. (D) Cells were fixed and the number of living cells as well as % of HDV-positive cells was determined by immunofluorescent staining and quantification with ImageJ. Results are the mean ± SD of 3 independent experiments (each dot represent the quantification of one field) and statistical analyses were performed using Mann-Whitney tests. (F) dHepaRG cells were coinfecting with HBV and HDV (HBV+HDV) with the indicated vge/cell. Cells were treated 6 days later (at the pic of HDV RNAs level) with BS1 (0.1 μg/ml) or Pam3CSK4 (0.1 μg/ml) for 11 days. Cells were collected and the levels of total intracellular HDV RNAs as well as the levels of HDV proteins were analysed by RT-qPCR and western blot, respectively. Results are the mean ± SD of 2 independent experiments each performed with 3 biological replicates and statistical analyses were performed using Mann-Whitney tests. (G) dHepaRG or freshly isolated PHHs were coinfecting with HBV (100 vge per cell) and with HDV (10 vge per cell). Cells were treated 3 days after with BS1 (0.5 μg/ml for dHepaRG or 2.5 μg/ml for PHHs) or Pam3CSK4 (0.5 μg/ml for dHepaRG or 2.5 μg/ml for PHHs) for 10 days.

activity – HDV replicates by hijacking host cellular enzymatic activities. Pegylated interferon- $\alpha$  (Peg-IFN $\alpha$ ) has been shown to suppress HDV viremia in a limited number of patients (14 to 50%) and relapses after arrest of treatment are very often reported.<sup>5-7</sup> Bulevirtide (previously called Myrcludex B), a peptide competing with HBV and HDV for cell entry via the NTCP (Na<sup>+</sup>-taurocholate cotransporting polypeptide) receptor, has proven its efficacy to also block HDV infection.<sup>8,9</sup> This drug very recently received an authorization for use in patients in Europe and is commercially available in France and Germany as Hepcludex. Only a few other antiviral strategies are currently under clinical evaluation for the treatment of chronic HDV infection. HDV assembly depends on the farnesylation of L-HDAG<sup>10</sup> and prenylation inhibitors were shown to inhibit HDV assembly and secretion. Results from the clinical trial to test the safety and effectiveness of Isonafarnib, in monotherapy or associated to Peg-IFN- $\lambda$ , are encouraging, but still limited by gastrointestinal side effects.<sup>11,12</sup> Nucleic acid polymers were also reported to potentially act on viral egress in patients.<sup>13,14</sup> None of these strategies (including bulevirtide) have been shown to target intracellular replication of the HDV genome, at least *in vitro*, and specific infectivity of produced HDV particles. The aim of our study was therefore to find alternative antiviral strategies preventing both HDV release and re-infection of hepatocytes.

We previously showed that hepatocytes (primary human hepatocytes [PHHs] and non-transformed differentiated HepaRG cells [dHepaRG]) express a number of innate immune receptors including Toll-like receptors (TLR) and the lymphotoxin  $\beta$  receptor (LT $\beta$ R)<sup>15,16</sup> that once activated, induce various inflammatory, anti-inflammatory, pro- and anti-survival pathways.<sup>17,18</sup> Moreover, cytokines such as IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6 as well as agonists of TLRs and LT $\beta$ R have been reported to control HBV in a non-cytopathic fashion.<sup>15,16,19-27</sup> In this study, we analyzed the effect of several immune-modulators on HDV in monoinfected or HBV/HDV-co/superinfected dHepaRG cells and PHHs.

## Materials and methods

### Cell culture and HBV infection

HepaRG cells were cultured, differentiated, and infected by HBV (genotype D) and HDV (genotype I) as previously described.<sup>28,29</sup> Infections (*i.e.* incubation of cells with the viruses) were performed overnight (between 12 h and 16 h). PHHs were freshly prepared from human liver resections obtained from the Centre Léon Bérard (Lyon) with French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011) as previously described.<sup>30</sup> HuH7.5-NTCP cells were cultivated in 10%-FCS supplemented DMEM (4.5 g/L glucose, Invitrogen). For infectivity assays, they were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> and infected 24 h later with HDV. Cells were maintained in medium complemented with 2% DMSO as soon as confluency was reached. HBV inocula were prepared from HepAD38 supernatants.<sup>31</sup> HDV inocula were prepared from supernatants from co-transfected HuH7 cells as previously described.<sup>29</sup> Supernatants

containing HBV or HDV particles were concentrated with 8% PEG 8000 (Sigma-Aldrich). All viral preparations were tested for the absence of endotoxin (Lonza). Concentrated viruses were characterized by analysis of the fractions from 20–44% iodixanol gradients. Of note, the rate of cell infection (dHepaRG and PHH) varies from one batch/donor to another. Data are therefore mostly presented as ratio to non-treated cells to be able to combine the different independent experiments.

For further details regarding the materials used, please refer to the [CTAT table and supplementary information](#).

## Results

### Antiviral effect on HDV of various immune-modulators

dHepaRG cells, infected for 7 days by HBV, then superinfected by HDV,<sup>29</sup> were treated (3 days post HDV infection) with Pam3CSK4 (TLR-1/2 agonist), BS1 (tetravalent bispecific antibody agonizing the LT $\beta$ R), a control IgG antibody, IFN- $\alpha$ , tenofovir (a nucleoside analogue inhibiting the reverse transcription of HBV) or with recombinant human (rh) IL-6 and rhTNF- $\alpha$ . As already reported<sup>15,21,22,26</sup> all those molecules, except the control IgG, decreased the levels of total intracellular HBV DNAs (Fig. 1A). IFN- $\alpha$  decreased the levels of total intracellular HBV DNA and HDV RNAs (Fig. 1A,B). However, the concentration used for the latter was at least 100 times higher than that used in humans (<sup>32</sup>; *in vivo* an equivalent of 10 IU/ml in the liver). Interestingly, a similar significant reduction of the levels of total intracellular HDV RNAs after treatment with Pam3CSK4, BS1, rhIL-6, and rhTNF- $\alpha$ , which are all related to NF- $\kappa$ B pathways, was also observed (Fig. 1B). Since the use of rhIL-6 and rhTNF- $\alpha$  as therapeutics by systemic injection will probably lead to severe adverse effects, we focused the rest of our study on Pam3CSK4 and BS1 that both interact with their respective receptors, which are expressed and would allow for the production of endogenous effectors by hepatocytes themselves, as previously described.<sup>15,16,22</sup>

### Pam3CSK4 and BS1 reduced the levels of intracellular HDV RNAs and proteins without toxicity

HDV-monoinfected, HBV/HDV-co/superinfected dHepaRG cells were treated with Pam3CSK4 and BS1 at the beginning of the HDV RNA amplification phase (we previously described the amplification phase being from day 3 to day 6 followed by a decrease in the levels of HDV RNAs already observed 9 days post-infection with HDV<sup>29</sup>). Upon stimulation of dHepaRG cells by Pam3CSK4 or BS1, we observed a reduction in the levels of total intracellular HDV RNA (50 to 75%) and HDV protein in all the different infection settings (Figs 1C,D and S1). Northern blot analyses revealed a reduction of both HDV-Gs and HDV-AGs upon treatment of infected cells with Pam3CSK4 and BS1 (Fig. 1E) suggesting that HDV RNA synthesis and/or HDV RNA stability may be targeted. The decreased levels of total intracellular HDV RNA and HDV protein observed upon treatment with Pam3CSK4 and BS1 were dose dependent (Fig. S2A,B) and still observed when increasing the multiplicity of infection of both

Supernatants were collected and release of LDH (assessing toxicity) were measured. Results are the mean  $\pm$  SD of 2 independent experiments (2 per cell type) each performed with 3 to 4 biological replicates. (H) Freshly isolated PHHs from 4 distinct donors were coinfecting with HBV (100 vge per cell) and with HDV (10 vge per cell). Cells were treated 3 days after with BS1 (1  $\mu$ g/ml) or Pam3CSK4 (1  $\mu$ g/ml) for 10 days. Cells were collected and the levels of total intracellular HDV RNAs were analysed by RT-qPCR. Results are the means of experiments performed with 4 donors with 3 or 4 biological replicates per experiment. dHepaRG, differentiated HepaRG; PHH, primary human hepatocyte; RT-qPCR, reverse-transcription quantitative PCR; vge, viral genome equivalent.

HBV and HDV, and when treatments started once HDV replication was at its maximum (6 days post-infection with HDV at the peak of HDV RNA levels) (Fig. 1F). As we reported already in HBV-infected dHepaRG cells,<sup>15,22</sup> we did not observe significant toxicity in HDV-monoinfected or HBV/HDV-superinfected dHepaRG cells treated with Pam3CSK4 and BS1 (Figs 1D,G and S2A,B). Since only a low number of dHepaRG cells replicate both viruses (Fig. 1D, S1A), cell death induced by Pam3CSK4 or BS1 specifically in infected cells might not be detected by regular cell survival assays. We therefore inhibited apoptosis using QVD-OPH (a pan caspase inhibitor<sup>33</sup>) and did not observe any alteration in the antiviral activity of either BS1 or Pam3CSK4 (Fig. S3). These data ruled out specific apoptosis of infected dHepaRG cells upon BS1 or Pam3CSK4 stimulation. As an alternative to the test all the other different types of cell death, we performed additional experiments with PHHs that are the gold standard for infectious assays and more susceptible to HBV and HDV infections than dHepaRG (Fig. S1A, B).<sup>29</sup> Given the limited supply of PHHs and since HDV associates to HBV in patients, we tested the effect of BS1 or Pam3CSK4 on HDV only in the setting of HBV/HDV coinfection and observed a reduction in the levels of total intracellular HDV RNAs in PHHs from 4 different donors (Fig. 1H) without toxicity (Figs 1G and S2C), demonstrating the clear antiviral effect of these agonists in the most relevant *in vitro* model. Of note, the presence of HDV did not abrogate the antiviral effect of Pam3CSK4 and BS1 that we already reported on HBV (Fig. S4).<sup>15,22</sup>

In order to assess the kinetics of HDV inhibition by Pam3CSK4 and BS1, HBV/HDV-superinfected dHepaRG cells were treated after establishment of the infection. Firstly, we monitored the levels of total intracellular HDV RNA under treatment. We observed 25% and 50% reductions in the levels of total intracellular HDV RNA 48 h and 96 h after the beginning of treatment, respectively (Fig. S5A). Secondly, as already described,<sup>15,22</sup> we observed a long-lasting antiviral effect of Pam3CSK4 and BS1 on the level of secreted HBV particles in contrast to treatment with a nucleoside analogue such as tenofovir, (Fig. S5B, right panel) and we did not observe any rebound in the levels of total intracellular HDV RNA up to 11 days after cessation of Pam3CSK4 or BS1 treatments (Fig. S5B, left panel).

#### **Pam3CSK4 and BS1 triggered the NF- $\kappa$ B pathways in HBV/HDV-coinfected dHepaRG cells**

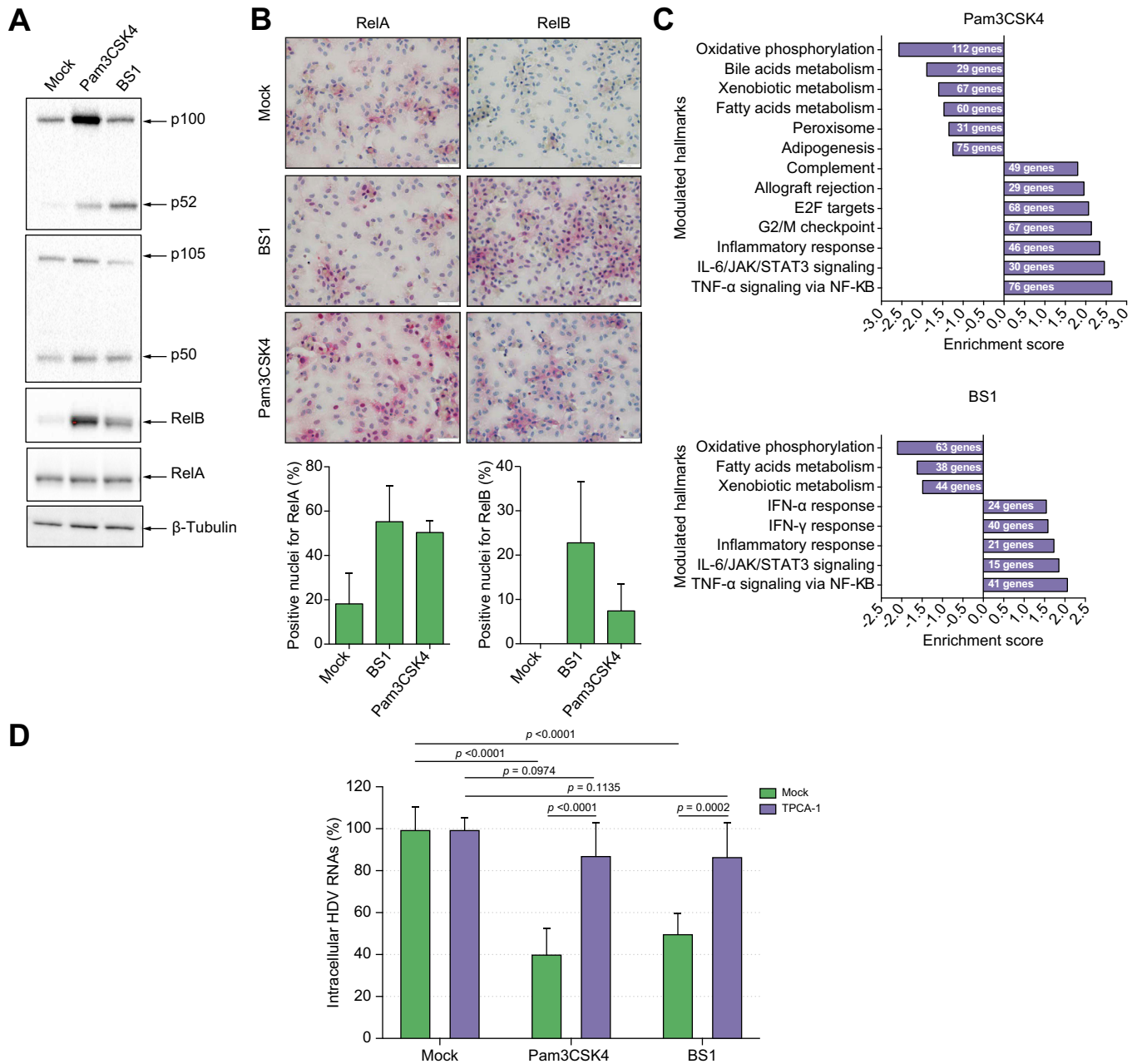
Stimulation of TLRs, as well as of LT $\beta$ R, is thought to induce various inflammatory pathways<sup>17,18</sup>; we aimed to confirm if this was still the case in HBV/HDV-coinfected dHepaRG cells. Western blot and cell staining showed that BS1 induced both the canonical and the alternative NF- $\kappa$ B pathways in HBV/HDV-coinfected dHepaRG cells (Fig. 2A,B) whereas Pam3CSK4 mainly induced the canonical NF- $\kappa$ B pathways as only very low levels of RelB nuclear translocation and p100 to p52 cleavage were observed (Fig. 2A,B). RNA sequencing was performed to gain an overview of the cell pathways modulated by each molecule. These analyses confirmed that genes of the NF- $\kappa$ B pathway were the most highly upregulated, followed by those involved in the IL6/JAK/STAT signaling pathway and inflammatory responses (Fig. 2C). Of note, metabolic pathways, such as fatty acid or xenobiotic metabolism pathways, were found to be negatively modulated by both ligands (Fig. 2C). Interestingly, TPCA-1, an IKK $\beta$  inhibitor we already used previously,<sup>34,35</sup>

abrogated the antiviral effect of Pam3CSK4 and BS1 on HDV (Fig. 2D).

In order to evaluate the quantity and quality of cytokines produced by dHepaRG cells upon stimulation by Pam3CSK4 or BS1, Luminex assays were performed 24 h after a first stimulation. None of the cytokines tested were detected in the supernatants of dHepaRG cells treated with BS1 (Fig. S6A). IL-6 was the only cytokine detected in the supernatants of HBV-infected dHepaRG cells treated by Pam3CSK4 (Fig. S6A). As we observed a decrease in the levels of intracellular HDV RNA in dHepaRG cells treated with rh-IL-6 (Fig. 1A), we performed experiments with neutralizing anti-IL-6 antibodies to assess the role of secreted IL-6 in the antiviral effect of Pam3CSK4 on HDV. Despite using an amount of anti-IL-6 antibodies able to neutralize more than 15 times the amount of IL-6 produced by dHepaRG cells upon Pam3CSK4 treatment (Fig. S6A), we did not observe any additional reduction in the levels of intracellular HDV RNA (Fig. S6B). We therefore concluded that IL-6 produced upon stimulation of dHepaRG cells by Pam3CSK4 is not responsible for the antiviral effect on HDV.

#### **Pam3CSK4 and BS1 reduced the infectivity of secreted HDV particles**

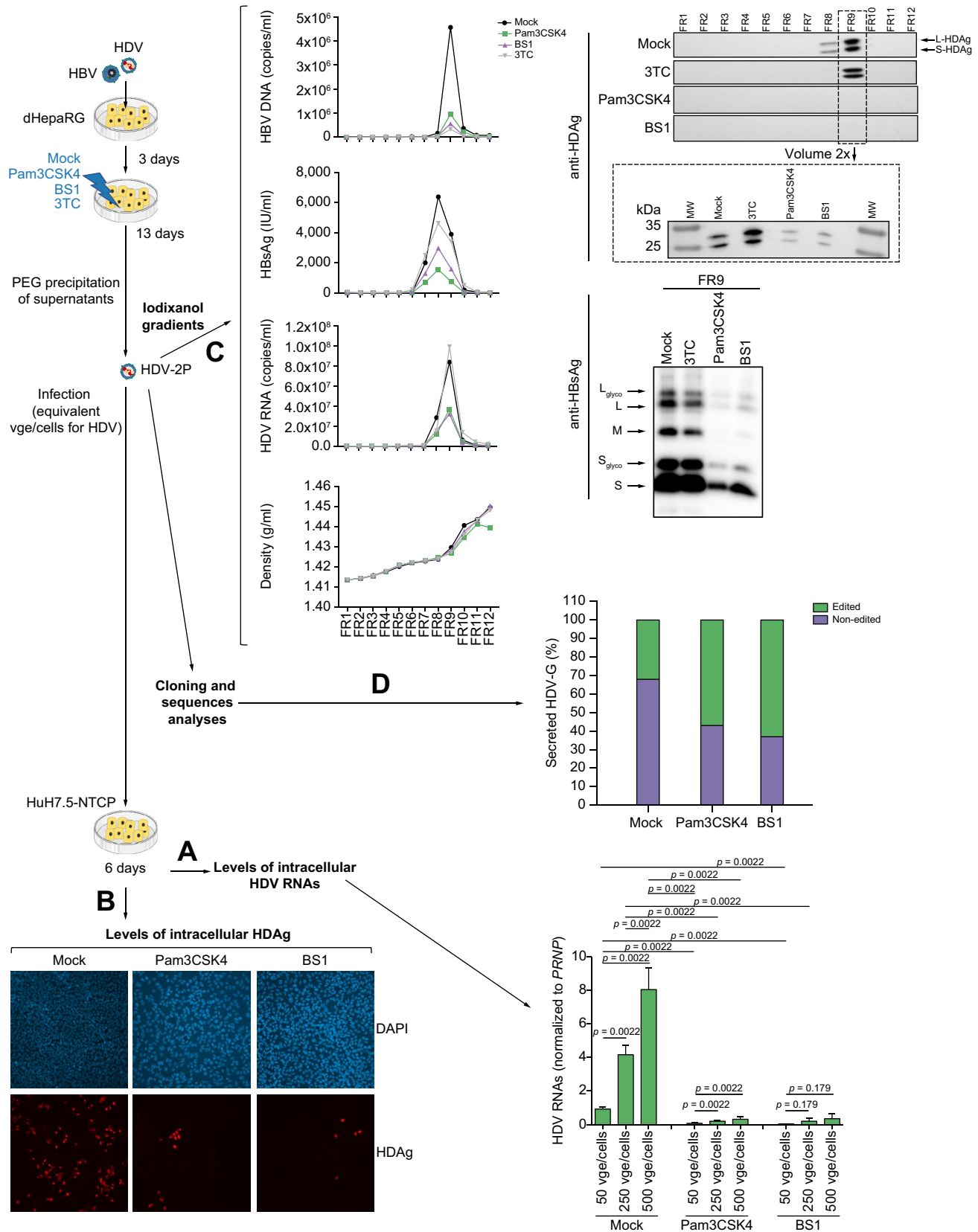
We next sought to evaluate the effect of Pam3CSK4 and BS1 on the secretion and infectivity of HDV particles, as viral spreading is the main mechanism leading to infection persistence in patients.<sup>36</sup> HBV/HDV-coinfected dHepaRG cells were treated with Pam3CSK4 and BS1 for 10 days and progeny viral particles quantified in the supernatants. We confirmed a reduction in the levels of intracellular HDAg upon treatments with Pam3CSK4 and BS1 (Fig. S7A). The supernatants of treated cells (containing progeny HBV and HDV, the latest being called HDV-2P) were collected and concentrated. Pam3CSK4 and BS1 also induced a reduction in the level of newly produced HDV particles (Fig. S7B). The different supernatants were subsequently used for inoculation of HuH7.5-NTCP cells (that, unlike dHepaRG cells, are highly susceptible to HDV infection, Fig. S1 and Fig. S8A) with an equal amount of viral genome equivalent (vge) per cell among the different conditions. The number of infected cells was assessed by immunofluorescence detection of HDAg as well as by quantifying intracellular HDV RNA 6 days after infection (Fig. 3A,B, S7C). We observed a strong decrease in the amount of HDAg-positive cells as well as in the levels of intracellular HDV RNAs when HuH7.5-NTCP cells were infected with HDV-2P particles produced by dHepaRG cells treated with Pam3CSK4 or BS1 compared to HuH7.5-NTCP cells infected with a similar amount of HDV-2P produced by non-treated dHepaRG cells (Fig. 3A,B, S7C). As a control, we confirmed that HDV-2P particles produced by dHepaRG cells treated with lamivudine (3TC), a nucleoside analogue that decreases HBV DNA synthesis without affecting HDV,<sup>29</sup> lead to similar levels of HDV RNA after infection of naïve HuH7.5-NTCP cells as with HDV-2P from non-treated dHepaRG cells (Fig. S7D). We excluded any possible effect on recipient cells of remaining molecules (Pam3CSK4 or BS1), most of which were likely titrated by the producing cells and eliminated during the concentration process of HDV-2P (PEG precipitation), since neither Pam3CSK4 nor BS1 affect HDV replication when added at the time of HuH7.5-NTCP cell infection (Fig. S8B). Moreover, probably due to very low expression of TLR2 in HuH7 cell lines,<sup>16</sup> Pam3CSK4, directly administered to HDV-infected HuH7.5-NTCP



**Fig. 2. Pam3CSK4 and BS1 on HDV triggered the activation of NF-κB pathways in HBV/HDV-coinfected dHepaRG cells.** (A) dHepaRG cells were coinfecting with HBV (100 vge per cell) and with HDV (10 vge per cell). Cells were treated 3 days later with BS1 (0.1 μg/ml) or Pam3CSK4 (0.1 μg/ml) for 10 days. Cells were collected and the levels of indicated proteins from the NF-κB pathways were analysed by western blot. (B) dHepaRG cells were coinfecting with HBV (100 vge per cell) and with HDV (10 vge per cell). Cells were treated 3 days later with BS1 (0.1 μg/ml) or Pam3CSK4 (0.1 μg/ml) for 3 days. Cells were fixed and RelA and RelB were stained using an alkaline phosphatase reaction. Positive signals are pink. Bars represent the mean ± SD from 2 independent experiments each performed using 4 fields for quantification. (C) dHepaRG cells were infected with HBV (100 vge per cell) and with HDV (10 vge per cell) 6 days later. Cells were treated 3 days after HDV infection with BS1 (0.1 μg/ml) or Pam3CSK4 (0.1 μg/ml) for 48 h. Cells were collected, RNA extracted and sequenced. Fold changes were calculated by comparison to non-treated cells and genes with a *p* value-adj <0.05 were selected and submitted to the GSEA software using the MSigDB data base. Molecular signatures are represented as bar-plots (FDR <0.25). (D) dHepaRG cells were infected with HDV with 50 vge/cell and treated 3 days with BS1 (0.1 μg/ml) or Pam3CSK4 (0.1 μg/ml) in the presence or not of TPCA-1 (10 uM) for 11 days. Cells were collected and the levels of total intracellular HDV RNAs were analysed by RT-qPCR. Results are the mean ± SD of 3 independent experiments each performed with 3 biological replicates and statistical analyses were performed using Mann-Whitney tests. dHepaRG, differentiated HepaRG; FDR, false discovery rate; GSEA, gene set-enrichment analysis; RT-qPCR, reverse-transcription quantitative PCR; vge, viral genome equivalent.

cells, did not affect the levels of HDV RNA (Fig. S8C), thus fully establishing that the loss of infectivity shown in Fig. 3A,B, S7C is due to an effect on viral progeny and specific infectivity. The

significant but lower antiviral effect on HDV of BS1 observed in HuH7.5-NTCP compared to dHepaRG might be due to their lack of differentiation (Fig. S8C).



**Fig. 3. Pam3CSK4 and BS1 reduced the infectivity of HDV particles.** dHepaRG cells were coinfecting with HBV (500 vge per cell) and with HDV (50 vge per cell). Cells were treated or not 3 days later with (A, B, C, D) Pam3CSK4 (0.1 µg/ml) or (A, B, C, D) BS1 (0.1 µg/ml) or (C) Lamivudine (3TC; 10 µM) for 10 days. Supernatants were collected, concentrated by PEG precipitation and the levels of extracellular HDV RNAs (called HDV-2P for second passage) were assessed by qRT-PCR analyses before (A, B) being used for infection of naïve HuH7.5-NTCP cells with the indicated vge per cell. Six days later, (A) levels of intracellular HDV RNA

To understand why we observed such a loss of infectivity of HDV-2P upon treatment of dHepaRG cells with Pam3CSK4 or BSA, we further analyzed HDV-2P by chemical approaches. HDV-2P particles produced by non-treated- or treated- dHepaRG cells were subjected to iodixanol gradients to analyze the ratios of S-HDAg/L-HDAg or S-HBsAg/L-HBsAg of HDV-2P that are essential for virion infectivity.<sup>4,37</sup> As already reported for circulating HDV virions in patients,<sup>38,39</sup> we found S-HDAg and L-HDAg in similar proportions in HDV-2P whether produced from dHepaRG non-treated or treated with Pam3CSK4 or BS1 (Fig. 3C). We also did not observe any major difference in the density of HDV-2P or in the ratios of S-HBsAg/L-HBsAg within HDV-2P for the different tested conditions (Fig. 3C). Of note, Pam3CSK4 and BS1 did not affect the association of HDAg to HDV RNA (assessed by RNA immunoprecipitation assay, Fig. S9) that is essential to form the HDV RNP in dHepaRG cells.<sup>4</sup> Altogether, these data suggest that the decreased infectivity of HDV-2P produced by dHepaRG cells treated with Pam3CSK4 or BS1 is not due to major defaults during HDV particle morphogenesis. Since both Pam3CSK4 and BS1 treatments of dHepaRG induce the expression of deaminases,<sup>15,22</sup> we next hypothesized that the decrease of infectivity could be due to hypermutations of secreted HDV-G RNAs. We therefore analyzed the HDV quasi-species of HDV-2P produced by dHepaRG cells after Pam3CSK4 or BS1 treatment followed by cloning of individual genomes and sequencing. Around 20 genomes were sequenced for each condition. We did not observe a major increase in the global mutation rate whether HDV-2P were produced in the presence of Pam3CSK4, BS1 or not (Fig. S10 and data not shown). However, when analyzing the amber termination codon usually edited by ADAR1 to produce L-HDAg,<sup>40</sup> we observed an increase in the ratio of edited vs. non-edited HDV-G secreted from dHepaRG cells treated with Pam3CSK4 or BS1 compared to non-treated cells (Fig. 3D, S10).

This difference in the ratio of secreted edited genomes occurred despite similar ADAR-1 levels in coinfecting dHepaRG cells treated with Pam3CSK4 or BS1 and mock-treated cells (Fig. S11).

## Discussion

Even though it leads to the most severe form of chronic viral hepatitis, HDV remains a neglected pathogen. Treatment options are still limited, despite the recent approval of bulevirtide in Europe – rates of sustained virological response off drug remain to be determined in real clinical practice. Peg-IFN- $\alpha$  remains a backbone of therapy despite inducing severe side effects. It would be important to find a therapeutic option, which could be at least as efficient as this combination of drugs. Herein, we showed, for the first time, that the stimulation of TLR-1/2 and LT $\beta$ R inhibits HDV through activation of the NF- $\kappa$ B pathway and independently of their effect on HBV replication.<sup>15,22</sup> Indeed, we observed that the stimulation of hepatocytes by ligands of TLR1/2 (Pam3CSK4) and LT $\beta$ R (BS1) prevents the amplification of intracellular HDV RNA and protein as efficiently as IFN- $\alpha$  and

without toxicity in HDV-replicating cells, as already reported for HBV-infected cells.<sup>15,22</sup> Both molecules also negatively affect HDV progeny release and strongly reduced their specific infectivity. We hypothesize that inhibition of intracellular HDV RNA amplification as well as decreased progeny-specific infectivity should strongly inhibit viral spread *in vivo* (that may either occur by re-infection of naïve cells or cell-to-cell spreading<sup>36</sup>).

Underlying mechanisms leading to intracellular reduction of HDV replication markers are likely to involve either negative epigenetic regulations of transcription from the HDV genome, direct degradation of HDV RNAs, editing with subsequent reduction in viral replication and/or dysregulation of metabolic pathways that might be essential for HDV (pathways of fatty acid metabolism were modulated by both ligands). Even though our neutralization assay suggests that the antiviral effect of Pam3CSK4 and BS1 appeared to be independent from IL-6 production and its secondary effect on hepatocytes via downstream pathways,<sup>41,42</sup> induction of this cytokine upon treatment with Pam3CSK4 may lead to a broader immune response *in vivo*. It is worth noting that the effect of Pam3CSK4 and BS1 on the levels of intracellular HBV RNAs was not equal in all PHH donors tested, suggesting that (i) either dHepaRG are particularly efficient at triggering innate cellular pathways, or (ii) the low level of replication of HDV in HepaRG is more prone to inhibition, and/or (iii) PHH donor heterogeneity (*e.g.* genetic background leading to variable expression of receptors, alteration of liver function due to the underlying donor disease or treatment) may affect pathways involved in HDV inhibition. This emphasizes the importance of performing experiments with multiple PHH donors and warrants further investigation in PHH *in vitro* with invigorated treatment (*e.g.* dosing every day for a longer period of time), as well as investigation in preclinical mouse models of HBV/HDV infection.<sup>43</sup>

The strongest antiviral effect of Pam3CSK4 and BS1 was observed at the level of HDV progeny infectivity and this is of particular interest since contrary to HBV that persists via the establishment of an episomal DNA within the nuclei of hepatocytes,<sup>44</sup> HDV is thought to be maintained in infected organisms by viral spreading (either by re-infection of naïve cells or cell-to-cell spreading).<sup>36</sup> Our analyses suggest that neither Pam3CSK4 nor BS1 induced a major defect in HDV particle morphogenesis (either in the ratio of S-HDAg and L-HDAg within the HDV RNP or of HBs isoforms in its envelope). However, we observed increased amounts of secreted HDV genome edited on the amber termination codon<sup>40</sup> upon treatment of dHepaRG cells with Pam3CSK4 or BS1. Following infection, such edited genomes would be responsible for the premature production of L-HDAg, resulting in the inhibition of HDV replication.<sup>4,37,45–47</sup> These observed changes in the ratio of secreted edited vs. non-edited genomes could, at least partially, explain the loss of infectivity of HDV particles produced upon treatments with Pam3CSK4 or BS1. Of note, the fact that neither the level of ADAR-1 (if we assumed that ADAR-1 is the sole protein responsible for edition of the amber codon) nor the

were assessed by RT-qPCR analyses or (B) cells were stained with DAPI and with anti-HDAg antibodies. Results are the mean  $\pm$  SD of two independent experiments each performed with 3 biological replicates and statistical analyses were performed using Mann-Whitney tests. (C) Concentrated supernatant were submitted to iodixanol gradients overnight. Fractions (FR) were collected and the levels of HDV RNA, HBV DNA, HBsAg, HDV protein were analysed by qRT-PCR, qPCR, ELISA and western blot respectively. (D) HDV RNAs were extracted from concentrated supernatants and sequences of HDV-G RNAs from HDV-2P were analysed after RT-PCR and cloning. Bars represent non-edited HDV-G and edited HDV-G at the amber termination codon (usually edited by ADAR1 to produce L-HDAg). See Fig. S9 for details of the sequencing. HDV-G, HDV genome; L-HDAg, large HDV antigen; MW, molecular weight; RT-qPCR, reverse-transcription quantitative PCR; vge, viral genome equivalent.

ratio of S-HDAg/L-HDAg are modified suggests that treatments with Pam3CSK4 or BS1 do not increase the editing *per se* but rather favor the packaging of edited HDV-G. Deep RNA sequencing of intracellular HDV RNAs should be performed to confirm this hypothesis and detailed mechanisms leading to this increased packaging of edited HDV-G upon treatments should be explored. Proteomic analyses of HDV virions produced upon these treatments may also enable us to uncover or rule out the additional role of host restriction factors that are upregulated and transported in the secreted HDV virions. Even though Pam3CSK4 and BS1 both lead to the activation of the canonical NF- $\kappa$ B pathway, it remains to be determined if the downstream activation/repression leading to their antiviral effect are similar. In particular, BS1 can also activate the non-canonical NF- $\kappa$ B pathway and some genes can be activated with particular kinetics as already reported.<sup>48</sup>

These findings have potential therapeutic implications. A TLR8 agonist (GS-9688), which is currently under clinical evaluation for the treatment of HBV, is also an inducer of NF- $\kappa$ B, but it induces this pathway only in TLR8-positive immune cells and not in hepatocytes that are negative for TLR8<sup>16,49</sup>; in this respect Pam3CSK4 would have broader efficacy. In contrast to agonists of TLR7 (e.g., GS-9620) and TLR8 (e.g., GS-9688) that can be delivered orally to humans, TLR2 ligands cannot and will likely

require specific strategies for liver-targeted delivery. In this respect, nanoparticles are currently being tested<sup>50</sup> to reduce the active dose of ligands, protect these ligands from degradation and specifically deliver them to the liver, thereby preventing systemic exposure and potential adverse effects.<sup>51</sup> These NPs-Pam3CSK4 will be tested in preclinical models of HBV/HDV infection to validate the antiviral concept *in vivo*, before moving to regulatory toxicological studies. Although a significant risk of inflammation and hepatocellular carcinoma has been associated with long and constitutive overexpression of LT $\alpha$ / $\beta$ ,<sup>52</sup> no adverse effects were observed with the use of LT $\beta$ R agonists for a limited timeframe in preclinical settings.<sup>53</sup>

In conclusion, besides being efficient against HBV,<sup>15,22</sup> we show here, for the first time, that immune-modulators inducing the NF- $\kappa$ B pathways in hepatocytes can also inhibit HDV replication and infectivity of progeny viruses *in vitro*. If proven to be effective *in vivo*, they could be further used in the development of treatments for chronically HBV/HDV-infected patients. Since HDV suppresses HBV replication in a majority of HBV/HDV-coinfected patients as well as in different animal models,<sup>54–64</sup> we herein propose new antiviral approaches targeting both HBV and HDV and thereby preventing any rebound of HBV replication that might be observed in HBV/HDV-coinfected patients with drugs only targeting HDV.

## Abbreviations

dHepaRG, differentiated HepaRG cells; HDV-AG(s), HDV anti-genome(s); HDV-G(s), HDV genome(s); IFN, interferon; IL-, interleukin-; L-HDAg, large HDV antigen; LT $\beta$ R, lymphotoxin- $\beta$  receptor; NTCP, Na<sup>+</sup>-taurocholate cotransporting polypeptide; Peg-IFN- $\alpha$ , pegylated interferon- $\alpha$ ; PHH, primary human hepatocyte; rh, recombinant human; RNP, ribonucleoprotein; S-HDAg, small HDV antigen; TLR, Toll-like receptor; TNF, tumor necrosis factor; vge, viral genome equivalent.

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## Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

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

## Authors' contributions

Study concept and design: JL, DD. Acquisition and analyses of data: JL, MM, DA, BC, CP, SFD, RF, TR, AFL, TE, AS. Interpretation of data: JL, MM, DA, BC, AS, DD. Drafting of the manuscript: JL, DA, AS, DD. Funding acquisition: JL, FZ, MH, DD. Material support: MR, MH, RR.

## Data availability statement

Data from RNA sequencing are available as described in the method part and [CTAT table](#). The rest of data presented in this manuscript are available through the corresponding author upon reasonable request.

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## Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2021.100415>.

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*Author names in bold designate shared co-first authorship*

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