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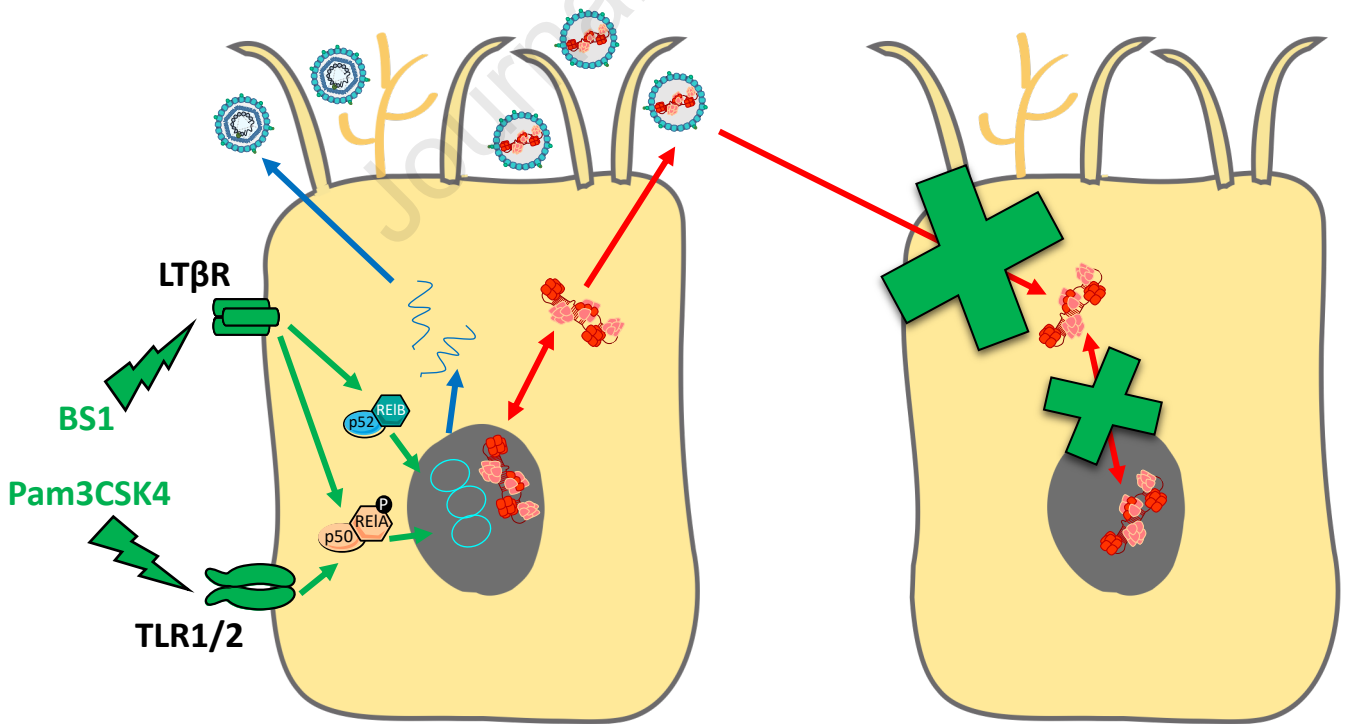
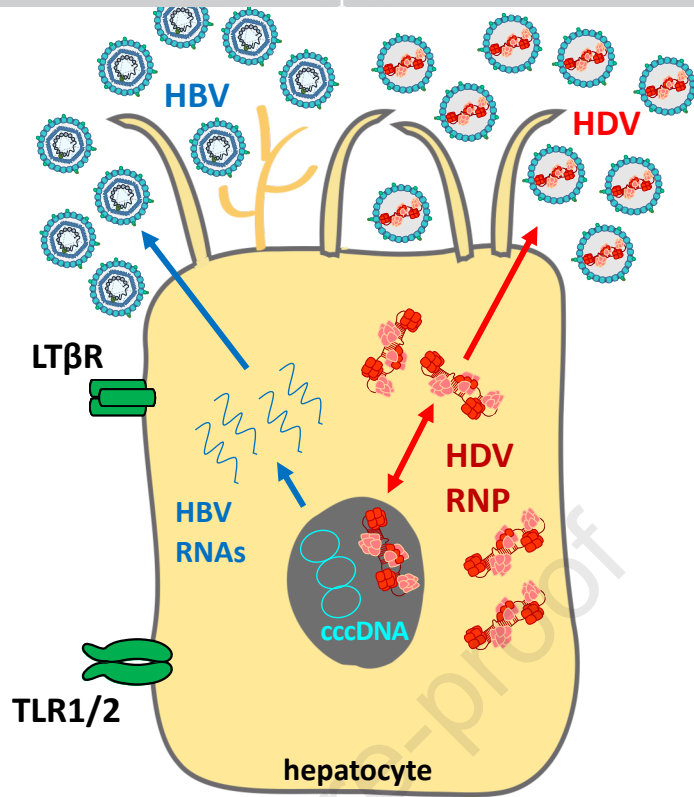
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**Pam3CSK4 or BS1**

- ↳ intracellular HBV and HDV RNAs
- ↳ secretion of HDV particles
- ↳ infectivity of HDV particles

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

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### Author contributions

- study concept and design: JL, DD
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- drafting of the manuscript: JL, DA, AS, DD
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- material support: MR, MH, RR

Journal Pre-proof

**Abstract**

**Background and aims:** Hepatitis Delta Virus (HDV) super-infection of chronically Hepatitis B Virus (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 IFN- $\alpha$  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 (PHH) 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 RNAs and of HDV proteins levels, without toxicity in cells mono infected with HDV or co/super-infected 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 human through constant propagation.

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

## 1 Introduction

2 Hepatitis D Virus (HDV)/Hepatitis B Virus co-infection is the most aggressive chronic form of  
3 viral hepatitis [1, 2] with an accelerated progression towards fibrosis/cirrhosis followed by  
4 clinical liver decompensation and related death [3]. HDV virions contain a ribonucleoprotein  
5 (RNP) composed of a circular single-stranded negative RNA genome presenting a “quasi”  
6 double-stranded conformation and viral proteins called HD antigens (HDAGs). HDV is a  
7 defective satellite virus that uses HBV envelope proteins to egress from and re-enter into  
8 hepatocytes. Apart from those steps, HDV intracellular RNA replication is known to be  
9 independent of HBV but is highly dependent on the cellular machinery (see [4] for review).  
10 Briefly, in the nucleus of infected cells, incoming HDV genomes (HDV-G) serve as template for  
11 the synthesis of replicative intermediates called anti-genomes (HDV-AG), which are fully  
12 complementary to HDV genome sequence and bear the open reading frame (ORF) for HDAG  
13 protein synthesis. S-HDAG is produced early after infection by transcription of HDV mRNAs  
14 from the incoming HDV-G within the HDV RNP and L-HDAG (that displays a C-terminal  
15 extension of 19 amino acids compared to S-HDAG) is produced later after editing of the amber  
16 termination codon on HDV-AG.

17 No direct acting agents, i.e. molecules specifically targeting viral enzymatic activities, against  
18 HDV are currently approved to treat chronic HDV infection because apart from the viral  
19 ribozyme activity, HDV replication occurs via hijacking of the cellular enzymatic activities.  
20 Pegylated interferon alpha (Peg-IFN $\alpha$ ) has been shown to suppress HDV viremia in a limited  
21 number of patients (14 to 50%) and relapses after arrest of treatment are very often reported  
22 [5-7]. Bulevirtide (previously called Myrcludex-B<sup>®</sup>), a peptide competing with HBV and HDV  
23 for cell entry via the NTCP (Na<sup>+</sup>-taurocholate cotransporting polypeptide) receptor, has  
24 proven its efficacy to also block HDV infection [8, 9]. This drug has very recently received an

25 authorization for use in patients in Europe and is commercially available in France and  
26 Germany as Hepcludex. Only few other antiviral strategies are currently under clinical  
27 evaluation for the treatment of chronic HDV infection. HDV assembly depends on the  
28 farnesylation of L-HDAg [10] and prenylation inhibitors were shown to inhibit HDV assembly  
29 and secretion. Results from the clinical trial to test the safety and effectiveness of Lonafarnib,  
30 in monotherapy or associated to Peg-IFN-lambda, are encouraging, but still limited by  
31 gastrointestinal side effects [11, 12]. Nucleic acids polymers were also reported to potentially  
32 act on viral egress in patients [13, 14]. None of these strategies (including Bulevirtide) have  
33 been shown to target intracellular replication of HDV genome, at least *in vitro*, and specific  
34 infectivity of produced HDV particles. The aim of our study was therefore to find alternative  
35 antiviral strategies preventing both HDV release and re-infection of hepatocytes.

36 We previously showed that hepatocytes (primary human hepatocytes (PHH) and non-  
37 transformed differentiated HepaRG cells (dHepaRG)) express a number of innate immune  
38 receptors including toll like receptors (TLR) and wells as the lymphotoxin  $\beta$  receptor (LT $\beta$ R)  
39 [15, 16] that once activated, induce various inflammatory, anti-inflammatory, pro- and anti-  
40 survival pathways [17, 18]. Moreover, cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-6 as well as agonists  
41 of TLRs and LT $\beta$ R have been described to control HBV in a non-cytopathic fashion [15, 16, 19-  
42 27]. In this study, we analyzed the effect of several immune-modulators on HDV in mono-  
43 infected or HBV/HDV co- or super-infected dHepaRG cell as well as primary human  
44 hepatocytes (PHH).



## 45 **Methods**

### 46 ***Cell culture and HBV infection***

47 HepaRG cells were cultured, differentiated, and infected by HBV (genotype D) and HDV  
48 (genotype I) as previously described [28, 29]. Infections (i.e incubation of cells with the viruses)  
49 were performed overnight (between 12 and 16h). PHH were freshly prepared from human  
50 liver resections obtained from the Centre Léon Bérard (Lyon) with French ministerial  
51 authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011) as previously  
52 described [30]. HuH7.5-NTCP cells were cultivated in 10%-FCS supplemented DMEM (4.5 g/L  
53 glucose, Invitrogen). For infectivity assays, they were seeded at  $2 \times 10^4$  cells/cm<sup>2</sup> and infected  
54 24h later with HDV. Cells were maintained in medium complemented with 2% DMSO as soon  
55 as confluency was reached. HBV inocula were prepared from HepAD38 supernatants [31].  
56 HDV inocula were prepared from supernatants from co-transfected HuH7 cells as previously  
57 described [29]. Supernatants containing HBV or HDV particles were concentrated with 8% PEG  
58 8000 (Sigma-Aldrich). All virus preparations were tested for the absence of endotoxin (Lonza).  
59 Concentrated viruses were characterized by analysis of the fractions from 20-44% iodixanol  
60 gradients. Of note, the rate of cells infection (dHepaRG and PHH) varies from one batch/donor  
61 to another. Data are therefore mostly presented as ratio to non-treated cells to be able to  
62 combine the different independent experiments.

63

### 64 **Reagents**

65 Pam3CSK4 (i.e. Pam3CysSerLys4, a synthetic triacylated lipopeptide and a TLR2/TLR1 ligand  
66 [32]) was purchased from InvivoGen, recombinant human IL-6 (rhIL-6) from R&D Systems, IFN-  
67  $\alpha$  (Roferon) from Roche and recombinant human TNF- $\alpha$  (rhTNF- $\alpha$ ) from Life Technologies.  
68 Neutralizing anti-IL-6 antibodies (IgG1, #501110) and their isotype control antibodies (IgG2,

69 #400414) were purchased from Biolegend. Tenofovir was obtained from Gilead Sciences.  
70 QVD-OPH (a pan-caspase inhibitor), Staurosporine (an inducer of apoptosis) and TPCA-1 were  
71 purchased from Sigma-Aldrich. To activate LT $\beta$ R, we used a super-agonistic tetravalent bi-  
72 specific antibody (BS1) [15, 33, 34]. If not indicated otherwise, cells were treated every 3-4  
73 days when media was changed.

74

#### 75 **Western blot and immunofluorescence analysis**

76 For analyses of total intracellular amount of proteins, cells were washed with Phosphate  
77 buffered saline (PBS) and harvested in RIPA lysis buffer (Tris-HCl pH 7.5 10 mM, NaCl 140 mM,  
78 EDTA 1 mM, EGTA 0.5 mM, 1% Triton X100, 0.1% SDS, 0.1% Na-Deoxycholate) containing  
79 protease inhibitors (Protein Cocktail Inhibitors from Sigma-Aldrich). For nuclear and cytoplasm  
80 separation, cells were lysed at 4°C in lysis buffer (PIPES 5 mM, KCL 85 mM, NP-40 0.5%)  
81 containing protease inhibitors with mechanic help (Dounce). After low speed centrifugation,  
82 supernatants corresponding to cytoplasm fraction were collected. Nuclear pellets were  
83 washed with lysis buffer before being lysed in RIPA lysis buffer.

84 Clarified lysates were subjected to SDS-PAGE and Western Blot transfer onto nitrocellulose  
85 membranes using the iBlot2 apparatus according to the manufacturer (Thermofisher  
86 Scientific). Detection was performed with Gel Doc XR+ System (BioRad) and images were  
87 analysed with ImageJ software. To perform immunofluorescence, cells were fixated with  
88 paraformaldehyde 4% and permeabilized by Triton 0.1%. Cell nuclei were stained with 4.6-  
89 diamidino-2-phenylindole (DAPI). Quantifications were done using ImageJ.

90 The anti-human Actin (clone C4, #08691002) was purchased from MP Biomedicals, the anti-  
91 human  $\beta$ Tubulin was purchased from Abcam. The polyclonal anti-HDAg serum, obtained by  
92 rabbit immunization (with a proprietary strategy), was a kind gift from Janssen. The anti-

93 p100/p52 (D7A9K), anti-p105/p50, anti-RelA, anti-Rel B (D7D7W), anti-ADAR-1 (81284) used  
94 for western blot analyses were purchased from Cell Signaling. The anti-HBsAg (H166) was  
95 previously described [35].

96

### 97 **Immunocytochemistry**

98 dHepaRG were seeded into 4-chamber slides (Thermo Fisher) at 350.000 cells/chamber. The  
99 next day, non-attached cells were removed, chambers washed once with PBS and cultured for  
100 3 more days in differentiation medium before infection. Experiments were stopped by  
101 removing culture medium, washing once with PBS and then incubating cells with 4%  
102 paraformaldehyde (Carl Roth) for 15 minutes at room temperature. Cells were then washed  
103 twice with PBS, before permeabilization with 0.2% Triton X-100 (Sigma Aldrich) for 10 minutes  
104 at room temperature. Cells were washed once with PBS, incubated 5 minutes in 70% ethanol  
105 and washed extensively in PBS again, before submitting to automated staining using the  
106 BOND-MAX Automated IHC/ISH Stainer (Leica) using RelA (Novus Biologicals, NB100-2176,  
107 1:200) or RelB (Cell Signaling Technologies, 4922, 1:200). Secondary antibody-polymer (Leica)  
108 coupled to alkaline phosphatase was used for detection.

109

### 110 ***Nucleic acid extractions, reverse transcription and qPCR analyses***

111 Total intracellular RNA and DNA were extracted from cells with the NucleoSpin RNA II kit and  
112 tissue kit respectively according to the manufacturer's instructions (Macherey-Nagel). DNAs  
113 and RNAs from HBV or HDV particles were isolated from the cell supernatants using the  
114 NucleoSpin RNA Virus kit (Macherey-Nagel) according to the manufacturer's instructions.  
115 RNA reverse transcription was performed using the Maxima RT (Life Technologies).  
116 Quantitative PCR for HDV and HBV were performed using specific primers and normalized to

117 *PRNP* housekeeping gene as previously described [15, 29].

118

### 119 ***Northern Blot analyses***

120 Northern blot for HDV detection was performed as previously described [29]. Membrane-  
121 bound RNA was hybridized to Digoxigenin(DIG)-labeled full HDV genome or anti-genome  
122 specific probes. 18S and/or 28S rRNA were used as loading control.

123

### 124 ***Measurement of cytokines by Luminex***

125 IL-12p70, GM-CSF, IFN- $\alpha$ , IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-13, IL-15, IL-18, IL-2, IL-5, IL-6, IL-7, and TNF- $\alpha$   
126 were measured with a ProcartaPlex Human Th1/Th2 & Cytokine Panel 1C (18 plex) kit  
127 (eBiosciences). Samples were analyzed using a Bio-Plex<sup>®</sup> 200 System (Bio-Rad, Hercules, CA)  
128 according to the manufacturer's instructions.

129

### 130 ***Viability/cytotoxicity assays***

131 Neutral red uptake assays were performed to estimate cell viability/cytotoxicity as previously  
132 described [36]. LDH release was assessed using the LDH-Glo cytotoxicity assay kit (Promega)  
133 according to the manufacturer's instruction. ATP levels were assessed using the CellTiter-Glo  
134 Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instruction.

135

### 136 ***RNA immunoprecipitation***

137 Cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Reaction was  
138 quenched with 0,125 M glycine for 5 min. Cells were lysed in RIPA buffer (see above) and  
139 clarified lysates were incubated with anti-HDAg Ab or the corresponding pre-immune sera  
140 (kind gift from Janssen) overnight at 4°C in a rotating wheel. Magnetic beads (Dynabeads™

141 Protein G, Thermofisher Scientific) were added and further incubated 2h with lysate and  
142 antibodies. Flow throughs (FT) were collected, beads washed with RIPA buffer. Half of the  
143 lysates/Ab/beads were washed with TE buffer (Tris-HCl pH 8 10mM, EDTA 10 mM) and RNAs  
144 were eluted by a 2h incubation at 68°C in elution buffer (Tris-HCl pH 7,5 20 mM; EDTA 5mM;  
145 NaCl 50 mM ; 1% SDS, Proteinase K 50 µg/mL). RNAs were then purified using Trizol based  
146 procedures. Flow through from the no antibody controls were also subjected to the same  
147 procedure and used as input for calculation.

148

### 149 **RNA sequencing and analyses**

150 RNAs were extracted with the miRNeasy Kit (Qiagen) according to the manufacturer's  
151 instruction. Library preparation for bulk 3'-sequencing of poly(A)-RNA was done as described  
152 previously [37]. The library was sequenced on a NextSeq 500 (Illumina) with 65 cycles for the  
153 cDNA in read1 and 16 cycles for the barcodes and UMIs in read2. Gencode gene annotations  
154 v28 and the human reference genome GRCh38 were derived from the Gencode homepage  
155 (EMBL-EBI). Dropseq tool v1.12 (Macosko et al., 2015) was used for mapping raw sequencing  
156 data to the reference genome. The resulting UMI filtered count matrix was imported into R  
157 v3.4.4. CPM (counts per million) values were calculated for the raw data and genes having a  
158 mean cpm value less than 1 were removed from the dataset. Prior differential expression  
159 analysis with DESeq2 v1.18.1 (10.1186/s13059-014-0550-8), dispersion of the data was  
160 estimated with a parametric fit using a dummy variable that codes all treatment-genotype  
161 combinations. The Wald test was used for determining differentially regulated genes between  
162 all treatments within a given genotype, as well as between genotypes for a particular  
163 treatment. Shrunk log<sub>2</sub> fold changes were calculated afterwards. A gene was determined  
164 to be differentially regulated if the absolute apeglm shrunk log<sub>2</sub> fold change was at least 1

165 and the adjusted p-value was below 0.01. GSEA v4.0.3  
166 (<https://doi.org/10.1073/pnas.0506580102>) was performed in the pre-ranked mode, where  
167 the apegln shrunken foldchange was used as ranking metric. All genes for which a test was  
168 conducted went into the analysis. Reference gene sets from the MsigDB v7.1  
169 (<https://doi.org/10.1093/bioinformatics/btr260>) were used for testing. A pathway was  
170 considered to be significantly associated with a genotype if the FDR value was below 0.05.  
171 Rlog transformation of the data was performed for visualization and further downstream  
172 analysis. Raw sequencing data are available under the accession number PRJEB39477.

173

#### 174 ***Analyses of HDV sequences released from HBV/HDV co-infection cells***

175 RNAs from HDV particles were isolated from the cell supernatants using the NucleoSpin RNA  
176 Virus kit (Macherey-Nagel) according to the manufacturer's instructions. RNA reverse  
177 transcription was performed using the Maxima RT (Life Technologies).

178 Cloning approaches: cDNAs were amplified using the HDVQ5<sup>®</sup> High-Fidelity DNA  
179 Polymerase (BioLabs) according to the manufacturer's instructions with the following primers:  
180 FW: CTTCCCGATGCTCGATTC; RV: GGCCGGAAGAAAGAAGTTAG. The resulting amplicons (from  
181 around 900 bp and covering the whole HDV ORF as well as ribozyme site) were cloned into  
182 pCR<sup>®</sup>-Blunt (Life Technologies) according to the manufacturer's instructions. DNAs from  
183 twenty positive clones were sent for sequencing (Eurofins) and analyzed with the SnapGene  
184 software.

185 -

186 **Results**

187 **Antiviral effect on HDV of various immune modulators.** dHepaRG cells, infected for 7 days by  
188 HBV, then super-infected by HDV [29], were treated (3 days post HDV infection) with  
189 Pam3CSK4 (TLR-1/2 agonist), BS1 (tetravalent bispecific antibody agonizing the LT $\beta$ R), a  
190 control IgG antibody, IFN- $\alpha$ , Tenofovir (a nucleoside analogue inhibiting the reverse  
191 transcription of HBV) or with recombinant human (rh) IL-6 and rhTNF- $\alpha$ . As already reported  
192 [15, 21, 22, 26] all those molecules, except the control IgG, decreased the levels of total  
193 intracellular HBV DNAs (**Figure 1A**). IFN- $\alpha$  decreased the levels of total intracellular HBV DNA  
194 and HDV RNAs (**Figure 1A, 1B**). However, the concentration used for the latter was at least  
195 100 times higher than that used in humans ([38]; *in vivo* an equivalent of 10 IU/ml in the liver).  
196 Interestingly, a similar significant reduction of the levels of total intracellular HDV RNAs after  
197 treatment with Pam3CSK4, BS1, rhIL-6, and rhTNF- $\alpha$ , which are all related to NF- $\kappa$ B pathways,  
198 was also observed (**Figure 1B**). Since the use of rhIL-6 and rhTNF- $\alpha$  as therapeutics by systemic  
199 injection, will probably lead to severe adverse effects, we focused the rest of our study on  
200 Pam3CSK4 and BS1 that both interact with their respective receptors, which are expressed  
201 and would allow the production of endogenous effectors by hepatocytes themselves, as  
202 previously described [15, 16] [22].

203  
204 **Pam3CSK4 and BS1 reduced the levels of intracellular HDV RNAs and proteins without**  
205 **toxicity.** HDV mono-infected, HBV/HDV co- or super-infected dHepaRG cells were treated with  
206 Pam3CSK4 and BS1 at the beginning of the HDV RNAs amplification phase (we previously  
207 described the amplification phase being from day 3 to day 6 followed by a decrease in the  
208 levels of HDV RNAs already observed 9 days post-infection with HDV [29]). Upon stimulation  
209 of dHepaRG cells by Pam3CSK4 or BS1, we observed a reduction in the levels of total

210 intracellular HDV RNAs (50 to 75 %) and HDV proteins in all the different infection settings  
211 **(Figure 1C, 1D, S1)**. Northern blot analyses revealed a reduction of both HDV-Gs and HDV-AGs  
212 upon treatment of infected cells with Pam3CSK4 and BS1 **(Figure 1E)** suggesting that HDV  
213 RNAs synthesis and/or HDV RNAs stability may be targeted. The decreased levels of total  
214 intracellular HDV RNAs and HDV proteins observed upon treatments with Pam3CSK4 and BS1  
215 were dose dependent **(Figure S2A, S2B)** and still observed when increasing the multiplicity of  
216 infection of both HBV and HDV, and when treatments started once HDV replication was at its  
217 maximum (6 days post-infection with HDV at the peak of HDV RNAs levels) **(Figure 1F)**. As we  
218 reported already in HBV-infected dHepaRG cells [15, 22], we did not observe significant  
219 toxicity in HDV mono-infected or HBV/HDV super-infected dHepaRG cells treated with  
220 Pam3CSK4 and BS1 **(Figure 1D, S2A, S2B, 1G)**. Since only a low number of dHepaRG cells  
221 replicate both viruses **(Figure 1D, S1A)**, cell death induced by Pam3CSK4 or BS1 specifically in  
222 infected cells might not be detected by regular cell survival assays. We therefore inhibited  
223 apoptosis using QVD-OPH (a pan caspase inhibitor [39]) and did not observe any alteration in  
224 the antiviral activity against HDV of neither BS1 nor Pam3CSK4 **(Figure S3)**. These data ruled  
225 out specific apoptosis of infected dHepaRG cells upon BS1 or Pam3CSK4 stimulation. As an  
226 alternative to the test all the other different types of cells death, we performed additional  
227 experiments with primary human hepatocytes (PHH) that are the gold standard for infectious  
228 assays and more susceptible to HBV and HDV infections than dHepaRG **(Figure S1A, S1B)** [29].  
229 Given the limited supply in PHH and since HDV associates to HBV in patients, we tested the  
230 effect of BS1 or Pam3CSK4 on HDV only in the HBV/HDV co-infection setting and observed a  
231 reduction in the levels of total intracellular HDV RNAs in PHH from four different donors  
232 **(Figure 1H)** without toxicity **(Figure 1G, S1C)**, demonstrating the clear antiviral effect of these  
233 agonists in the most relevant *in vitro* model. Of note, the presence of HDV did not abrogate



234 the antiviral effect of Pam3CSK4 and BS1 that we already reported on HBV (**Figure S4**) [15,  
235 22].

236 In order to assess the kinetics of HDV inhibition by Pam3CSK4 and BS1, HBV/HDV super-  
237 infected dHepaRG cells were treated after establishment of the infection. Firstly, we  
238 monitored the levels of total intracellular HDV RNAs under treatment. We observed 25% and  
239 50% reduction of the levels of total intracellular HDV RNAs 48h and 96h after the beginning of  
240 treatment respectively (**Figure S5A**). Secondly, as already described [15, 22], we observed a  
241 long-lasting antiviral effect of Pam3CSK4 and BS1 on the level of secreted HBV particles in  
242 contrast to treatment with a nucleoside analogue such as Tenofovir, (**Figure S5B, right panel**)  
243 and we did not observe any rebound in the levels of total intracellular HDV RNAs up to 11 days  
244 after arrest of Pam3CSK4 or BS1 treatments (**Figure S5B, left panel**).

245

246 **Pam3CSK4 and BS1 triggered the NF- $\kappa$ B pathways in HBV/HDV co-infected dHepaRG cells.**

247 Stimulation of TLRs as well as the of LT $\beta$ R are thought to induce various inflammatory  
248 pathways [17, 18] and we aimed to confirm it was still the case in HBV/HDV co-infected  
249 dHepaRG cells. Western Blot and cell staining showed that BS1 induced both the canonical  
250 and the alternative NF- $\kappa$ B pathways in HBV/HDV co-infected dHepaRG cells (**Figures 2A and**  
251 **2B**) whereas Pam3CSK4 mainly induced the canonical NF- $\kappa$ B pathways as only very low levels  
252 of RelB nuclear translocation and p100 to p52 cleavage were observed (**Figures 2A and 2B**).  
253 RNA sequencing was performed to have an overview of the cell pathways modulated by each  
254 molecule. These analyses confirmed that genes of the NF- $\kappa$ B pathway were the most highly  
255 upregulated followed by those involved in the IL6/JAK/STAT signaling pathway and  
256 inflammatory responses (**Figures 2C**). Of note, metabolic pathways such as fatty acids or  
257 xenobiotic metabolism pathways were found negatively modulated by both ligands (**Figure**

258 **2C)**. Interestingly, TPCA-1, an IKK $\beta$  inhibitor we already used previously [40, 41], abrogated the  
259 antiviral effect of Pam3CSK4 and BS1 on HDV (**Figure 2D**).

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

272  
273 ***Pam3CSK4 and BS1 reduced the infectivity of secreted HDV particles.*** We next sought to  
274 evaluate the effect of Pam3CSK4 and BS1 on the secretion and infectivity of HDV particles, as  
275 viral spreading is the main mechanism leading to infection persistence in patients [42].  
276 HBV/HDV co-infected dHepaRG cells were treated with Pam3CSK4 and BS1 for 10 days and  
277 progeny viral particles quantified in the supernatants. We confirmed a reduction in the levels  
278 of intracellular HDAg upon treatments with Pam3CSK4 and BS1 (**Figure S7A**). The supernatants  
279 of treated cells (containing progeny HBV and HDV, the latest being called HDV-2P) were  
280 collected and concentrated. Pam3CSK4 and BS1 also induced a reduction in the level of newly  
281 produced HDV particles (**Figure S7B**). The different supernatants were subsequently used for

282 inoculation of HuH7.5-NTCP cells (that, unlike dHepaRG cells, are highly susceptible to HDV  
283 infection, **Figure S1** and **Figure S8A**) with an equal amount of vge per cell among the different  
284 conditions. The number of infected cells was assessed by immunofluorescence detection of  
285 HDAg as well as by quantifying intracellular HDV RNA 6 days after infection (**Figure 3A, 3B**).

286 We observed a strong decrease in the amount of HDAg positive cells as well as in the levels of  
287 intracellular HDV RNAs when HuH7.5-NTCP cells were infected with HDV-2P particles  
288 produced by dHepaRG cells treated with Pam3CSK4 or BS1 compared to HuH7.5-NTCP cells  
289 infected with a similar amount of HDV-2P produced by non-treated dHepaRG cells (**Figure 3A,**  
290 **3B**). As a control, we confirmed that HDV-2P particles produced by dHepaRG cells treated with  
291 Lamivudine (3TC), a nucleoside analogue that decrease HBV DNA synthesis without affecting  
292 HDV [29], lead to similar levels of HDV RNAs after infection of naïve HuH7.5-NTCP cells as with  
293 HDV-2P from non-treated dHepaRG cells (**Figure S7D**). We excluded any possible effect on  
294 recipient cells of remaining molecules (Pam3CSK4 or BS1), most of which were likely titrated  
295 by the producing cells and eliminated during the concentration process of HDV-2P (PEG  
296 precipitation), since neither Pam3CSK4, nor BS1 affect HDV replication when added at the  
297 time of HuH7.5-NTCP cells infection (**Figure S8B**). Moreover, probably due to very low  
298 expression of TLR2 in HuH7 cell lines [16], Pam3CSK4, directly administrated to in HDV-  
299 infected HuH7.5-NTCP cells, did not affect the levels of HDV RNAs (**Figure S8C**), thus fully  
300 establishing that the loss of infectivity showed in **Figures 3A, 3B** is due to an effect on viral  
301 progeny and specific infectivity. The significant but lower antiviral effect on HDV of BS1  
302 observed in HuH7.5-NTCP compared to dHepaRG might be due the lack of differentiation of  
303 the cells (**Figure S8C**).

304 To understand why we observed such a loss of infectivity of HDV-2P upon treatment of  
305 dHepaRG cells with Pam3CSK4 or BSA, we further analyzed HDV-2P by chemical approaches.

306 HDV-2P particles produced by non-treated- or treated- dHepaRG cells were subjected to  
307 iodixanol gradients to analyze the ratios of S-HDAg/L-HDAg or S-HBsAg/L-HBsAg of HDV-2P  
308 that are essential for virion infectivity [4, 43]. As already reported for circulating HDV virions  
309 in patients [44, 45], we found S-HDAg and L-HDAg in similar proportions in HDV-2P whether  
310 produced from dHepaRG non-treated or treated with Pam3CSK4 or BS1 (**Figure 3C**). We also  
311 did not observe any major difference neither in the density of HDV-2P, nor in the ratios of S-  
312 HBsAg/L-HBsAg within HDV-2P for the different tested conditions (**Figure 3C**). Of note,  
313 Pam3CSK4 and BS1 did not affect the association of HDAg to HDV RNAs in dHepaRG cells  
314 (assessed by RNA immunoprecipitation assay, **Figure S9**), that is essential to form the HDV  
315 ribonucleoprotein [4]. Altogether, these data suggest that the decrease of infectivity of HDV-  
316 2P produced by dHepaRG cells treated with Pam3CSK4 or BS1 is not due to major defaults  
317 during HDV particles morphogenesis. Since both Pam3CSK4 and BS1 treatments of dHepaRG  
318 induce the expression of deaminases [15, 22], we next hypothesized that the decrease of  
319 infectivity could be due to hypermutations of secreted HDV-G RNAs. We therefore analyzed  
320 the HDV quasi-species of HDV-2P produced by dHepaRG cells after Pam3CSK4 or BS1  
321 treatment followed by cloning of individual genomes and sequencing. Around twenty  
322 genomes were sequenced for each condition. We did not observe a major increase in the  
323 global mutation rate whether HDV-2P were produced in the presence of Pam3CSK4, BS1 or  
324 not (**Figure S10** and data not shown). However, when analyzing the amber termination codon  
325 usually edited by ADAR1 to produce L-HDAg [46], we observed an increase in the ratio of  
326 edited versus non-edited HDV-G secreted from dHepaRG cells treated with Pam3CSK4 or BS1  
327 compared to non-treated cells (**Figure 3D, S10**).

328 This difference in the ratio of secreted edited genomes occurred despite similar ADAR-1 levels  
329 in co-infected dHepaRG cells treated with Pam3CSK4 or BS1 and mock-treated cells  
330 **(Figure S11)**.

331

## 332 **Discussion**

333 Even though it leads to the most severe form of chronic viral hepatitis, HDV remains a  
334 neglected pathogen. Treatment options are still limited, despite the recent approval of  
335 Bulevirtide in Europe for which it remains to be determined in real clinical practice what will  
336 be the sustained virological response off-drug. Peg-IFN- $\alpha$  remains a backbone of the therapy  
337 despite inducing severe side effects. It would be important to find a therapeutic option, which  
338 could be at least as efficient as this combination of drugs. Here we showed, for the first time,  
339 that the stimulation of TLR-1/2 and LT $\beta$ R inhibits HDV through activation of the NF $\kappa$ B pathway  
340 and independently of their effect on HBV replication [15, 22]. Indeed, we observed that the  
341 stimulation of hepatocytes by ligands of TLR1/2 (Pam3CSK4) and LT $\beta$ R (BS1) prevents the  
342 amplification of intracellular HDV RNAs and proteins as efficiently as IFN- $\alpha$  and without  
343 toxicity in HDV replicating cells as already reported for HBV infected cells [15, 22]. Both  
344 molecules also negatively affect HDV progeny release and strongly reduced their specific  
345 infectivity. We hypothesize that inhibition of intracellular HDV RNAs amplification as well as  
346 decrease of progeny specific infectivity should strongly inhibit viral spread *in vivo* (that may  
347 either occur by re-infection of naïve cells or cell-to-cell spreading [42]).

348 Underlying mechanisms leading to intracellular reduction of HDV replication markers are likely  
349 to involve either negative epigenetic regulations of the transcription from HDV genome, direct  
350 degradation of HDV RNAs, editing with subsequent reduction in viral replication and/or  
351 dysregulation of metabolic pathways that might be essential for HDV as fatty acids

352 metabolism pathways were found modulated by both ligands. Even though our neutralization  
353 assay suggests that the antiviral effect of Pam3CSK4 and BS1 appeared to be independent  
354 from IL-6 production and its secondary effect on hepatocytes via downstream pathways [47,  
355 48], induction of this cytokine upon treatment with Pam3CSK4 may lead to a broader immune  
356 response *in vivo*. It is worth noting that the effect of Pam3CSK4 and BS1 on the levels of  
357 intracellular HBV RNAs was not equal in all PHH donors tested suggesting that (i) either  
358 dHepaRG are particularly efficient to trigger innate cellular pathways, or (ii) the low level of  
359 replication of HDV in HepaRG is more prone to inhibition, and/or (iii) PHH donor heterogeneity  
360 (*e.g.* genetic background leading to variable expression of receptors, alteration of liver  
361 function due the underlying donor disease or treatment) may affect pathways involved in HDV  
362 inhibition. This emphasizes the importance of performing experiments with multiple PHH  
363 donors and warrants further investigation in PHH *in vitro* with invigorated treatment (*e.g.*  
364 dosing every day for a longer period of time), as well as investigation in preclinical mouse  
365 models of HBV/HDV infection [49].

366 The strongest antiviral effect of Pam3CSK4 and BS1 was observed at the level of HDV progeny  
367 infectivity and this is of particular interest since contrary to HBV that persists via the  
368 establishment of an episomal DNA within the nuclei of hepatocytes [50], HDV is thought to be  
369 maintained in infected organisms by viral spreading (either by re-infection of naïve cells or cell  
370 to cell spreading) [42]. Our analyses suggest that neither Pam3CSK4 nor BS1 induced major  
371 default in HDV particles morphogenesis (either in the ratio of S-HDAg and L-HDAg within the  
372 HDV RNP or of HBs isoforms in its envelope). However, we observed increased amounts of  
373 secreted HDV genome edited on the amber termination codon [46] upon treatment of  
374 dHepaRG cells with Pam3CSK4 or BS1. Following infection, such edited genomes would be  
375 responsible of a premature production of L-HDAg, resulting in the inhibition HDV replication

376 [4, 43, 51-53]. These observed changes in the ratio of secreted edited versus non-edited  
377 genomes could, at least partially, explain the loss of infectivity of HDV particles produced upon  
378 treatments with Pam3CSK4 or BS1. Of note, the fact that neither the level of ADAR-1 (if we  
379 assumed that ADAR-1 is the sole protein responsible for edition of the amber codon) nor the  
380 ratio of S-HDAg/L-HDAg are modified suggests that treatments with Pam3CSK4 or BS1 do not  
381 increase the editing per se but rather favor the packaging of edited HDV-G. Deep RNA  
382 sequencing of intracellular HDV RNAs should be performed to confirm this hypothesis and  
383 detailed mechanisms leading to this increased packaging of edited HDV-G upon treatments  
384 should be explored. Proteomic analyses of HDV virions produced upon these treatments may  
385 also allow to uncover or rule out the additional role of host restriction-factors being  
386 upregulated and embarked in the secreted HDV virions. Even though Pam3CSK4 and BS1 both  
387 lead to the activation of the canonical NF- $\kappa$ B pathway, it remains to be determined if the  
388 downstream activation/repression leading to their antiviral effect are similar. In particular  
389 because BS1 can also activate the non-canonical NF- $\kappa$ B pathway and kinetic of some genes  
390 activation might be particular as already reported [54].

391 These findings have potential therapeutic implications. TLR8 agonist (e.g., GS-9688), which is  
392 currently clinically evaluated against HBV, is also an inducer of NF- $\kappa$ B, but it induces this  
393 pathway only in TLR8-positive immune cells and not in hepatocytes that are negative for TLR8  
394 [16, 55]; in this respect Pam3CSK4 would have a broaden efficacy. In contrast to agonists of  
395 TLR7 (e.g., GS-9620) and TLR8 (e.g., GS-9688) that can be delivered orally to human, TLR2  
396 ligands cannot and will likely require specific strategies for liver-targeted delivery. In this  
397 respect, nanoparticles are currently tested [56] to reduce the active dose of ligands, protect  
398 these ligands from degradation and specifically deliver them to the liver, thereby preventing  
399 systemic exposure and potential adverse effects [57]. These NPs-Pam3CSK4 will be tested in

400 preclinical models of HBV/HDV infection to validate the antiviral concept *in vivo*, before  
401 moving to regulatory toxicological studies. Although a significant risk of inflammation and  
402 hepatocellular carcinoma has been associated to long and constitutive overexpression of  
403  $LT\alpha/\beta$  [58], no adverse effects were observed with the use of  $LT\beta R$ -agonists for a limited time  
404 frame in preclinical settings [33].

405 In conclusion, besides being efficient against HBV [15, 22], we show here, for the first time,  
406 that immune-modulators inducing the NF- $\kappa B$  pathways in hepatocytes can also inhibit HDV  
407 replication and infectivity of progeny viruses *in vitro*. If proven to also be efficient *in vivo*, they  
408 could be further used in the development of treatment for HBV/HDV chronically infected  
409 patients. Since HDV suppresses HBV replication in a majority of HBV/HDV co-infected patients  
410 as well as in different animal models [59-69], we propose here new antiviral approaches  
411 targeting both HBV and HDV and thereby preventing any rebound of HBV replication that  
412 might be observed in HBV/HDV co-infected patients with drugs only targeting HDV.

413

#### 414 **List of abbreviations**

415 4,6-diamidino-2-phenylindole, DAPI;  
416 antigen, Ag;  
417 differentiated HepaRG cells, dHepaRG;  
418 Digoxigenin, DIG;  
419 direct acting agents, DAA;  
420 dimethylsulfoxide, DMSO;  
421 DNA-dependent/RNA-polymerase II, RNA Pol-II ;  
422 Fecal calf serum, FCS;  
423 hepatitis B virus, HBV;  
424 hepatitis C virus, HCV;  
425 hepatitis D virus, HDV;  
426 host targeting agents, HTA;  
427 human immunodeficiency virus, HIV;  
428 interferon, IFN;  
429 interleukin, IL;  
430 lymphotoxin-beta receptor,  $LT\beta R$ ;  
431  $Na^+$ -taurocholate cotransporting polypeptide, NTCP;  
432 nuclear factor-kappa B, NF- $\kappa B$



433 open reading frame, ORF;  
434 Phosphate buffered saline, PBS;  
435 pegylated interferon alpha, Peg-IFN- $\alpha$ ;  
436 primary human hepatocyte, PHH;  
437 ribonucleoprotein, RNP;  
438 toll-like receptor, TLR;  
439 tumor necrosis factor alpha ; TNF- $\alpha$ ,  
440 viral genome equivalent, vge;  
441 World Health Organization, WHO;  
442

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448

449

450 **Data Availability Statement:** Data from RNA sequencing are available as described in the  
451 method part and CTAT table. The rest of data presented in this manuscript are available  
452 through the corresponding author upon reasonable request

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454 Author names in bold designate shared co-first authorship

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630 **Figures legends**

631

632 **Figure 1: Inducers of the NF- $\kappa$ B pathway such as Pam3CSK4 and BS1 reduced the levels of**633 **intracellular HDV RNAs and proteins.** (A, B) dHepaRG cells were infected with HBV and then

634 with HDV 6 days later with 100 vge/mL per cell for HBV and 10 vge/mL per cell for HDV. Cells

635 were treated 3 days after HDV infection with BS1 (0.5  $\mu$ g/mL) or Pam3CSK4 (0.5  $\mu$ g/mL), IFN-636  $\alpha$  (1000 U/mL), control IgG (0.5  $\mu$ g/mL), Tenofovir (10  $\mu$ M), TNF- $\alpha$  (50 ng/mL) or recombinant

637 human IL-6 (rhIL-6, 100 ng/mL) for 11 days. Cells were collected and the levels of total

638 intracellular (A) HBV DNAs and (B) HDV RNAs were analyzed by RT-qPCR and qPCR

639 respectively. Results are the mean  $\pm$  SD of 2 (for HBV RNAs) or 3 (for HDV RNAs) independent

640 experiments each performed with three biological replicates and statistical analyses were

641 performed using Mann-Whitney tests. (C,D,E) dHepaRG cells were infected with HDV (HDV),

642 HBV and with HDV 6 days later (HBV-&gt;HDV) or co-infected with HBV and HDV (HBV+HDV) with

643 100 vge per cell for HBV and 10 vge per cell for HDV. Cells were treated 3 days after HDV

644 infection with (C, D) BS1 (0.1  $\mu$ g/mL), Pam3CSK4 (0.1  $\mu$ g/mL) or (E) IFN- $\alpha$  (1000 U/mL) for 11

645 days. Cells were collected and the levels of total (C, E) intracellular HDV RNAs as well as the

646 levels (C) HDV proteins were analyzed by (C) RT-qPCR, (E) Northern Blot or (C) Western-blot

647 respectively. (C) Results are the mean  $\pm$  SD of 2 independent experiments each performed

648 with four biological replicates and statistical analyses were performed using Mann-Whitney

649 tests. (D) Cells were fixed and the number of living cells as well as % of HDV positive cells was

650 determined by immunofluorescent staining and quantification with ImageJ. Results are the

651 mean  $\pm$  SD of 3 independent experiments (each dot represent the quantification of one field)

652 and statistical analyses were performed using Mann-Whitney tests. (F) dHepaRG cells were

653 co-infected with HBV and HDV (HBV+HDV) with the indicated vge/cell. Cells were treated 6

654 days later (at the pic of HDV RNAs level) with BS1 (0.1 µg/mL) or Pam3CSK4 (0.1 µg/mL) for  
655 11 days. Cells were collected and the levels of total intracellular HDV RNAs as well as the levels  
656 of HDV proteins were analysed by RT-qPCR or Western-blot respectively. Results are the mean  
657 +/- SD of 2 independent experiments each performed with three biological replicates and  
658 statistical analyses were performed using Mann-Whitney tests. (G) Freshly isolated PHH from  
659 four distinct donors were co-infected with HBV (100 vge per cell) and with HDV (10 vge per  
660 cell). Cells were treated 3 days after with BS1 (1 µg/mL) or Pam3CSK4 (1 µg/mL) for 10 days.  
661 Cells were collected and the levels of total intracellular HDV RNAs were analysed by RT-qPCR.  
662 Results are the means of experiments performed with four donors with three or four biological  
663 replicates per experiment. (H) dHepaRG or freshly isolated PHH were co-infected with HBV  
664 (100 vge per cell) and with HDV (10 vge per cell). Cells were treated 3 days after with BS1 (0.5  
665 µg/mL for dHepaRG ou 2.5 µg/mL for PHH) or Pam3CSK4 (0.5 µg/mL for dHepaRG ou 2.5  
666 µg/mL for PHH) for 10 days. Supernatants were collected and release of LHD (assessing  
667 toxicity) were measured. Results are the mean +/- SD of 2 independent experiments (2 per  
668 cell type) each performed with three to four biological replicates.

669

670 **Figure 2: Pam3CSK4 and BS1 on HDV triggered the activation of NF-κB pathways in HBV/HDV**  
671 **co-infected dHepaRG cells.** (A) dHepaRG cells were co-infected with HBV (100 vge per cell)  
672 and with HDV (10 vge per cell). Cells were treated 3 days later with BS1 (0.1 µg/mL) or  
673 Pam3CSK4 (0.1 µg/mL) for 10 days. Cells were collected and the levels of indicated proteins  
674 from the NF-κB pathways were analysed by Western-blot. (B) dHepaRG cells were co-infected  
675 with HBV (100 vge per cell) and with HDV (10 vge per cell). Cells were treated 3 days later with  
676 BS1 (0.1 µg/mL) or Pam3CSK4 (0.1 µg/mL) for 3 days. Cells were fixed and RelA and RelB were  
677 stained using an alkaline phosphatase reaction. Positive signals are pink. Bars represent the

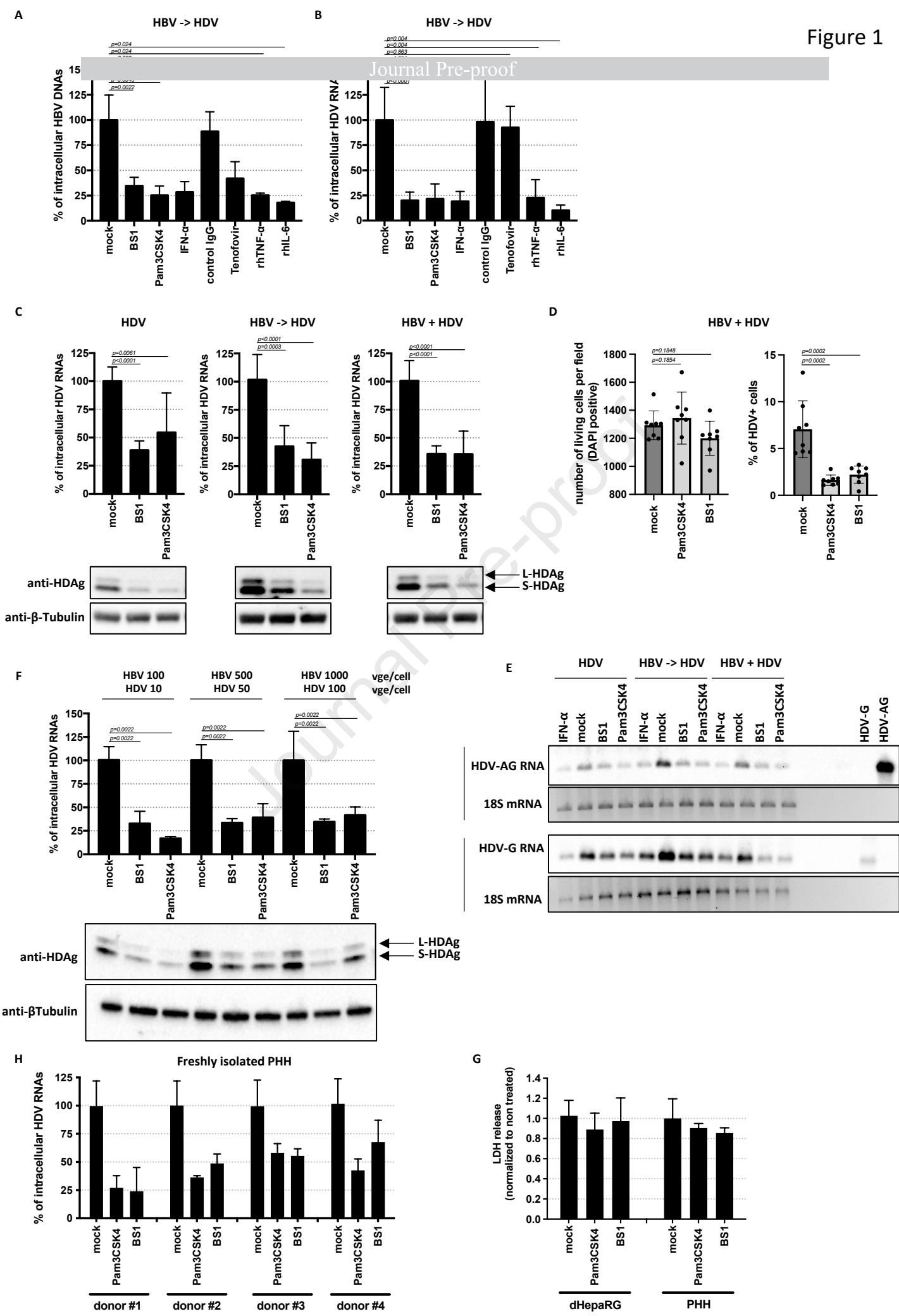
678 mean +/- SD from 2 independent experiments each performed using 4 fields for  
679 quantification. (C) dHepaRG cells were infected with HBV (100 vge per cell) and with HDV (10  
680 vge per cell) 6 days later. Cells were treated 3 days after HDV infection with BS1 (0.1 µg/mL)  
681 or Pam3CSK4 (0.1 µg/mL) for 48h. Cells were collected, RNA extracted and sequenced. Fold  
682 changes were calculated by comparison to non-treated cells and genes with a p-value-adj  
683 <0,05 were selected and submitted to the GSEA software using the MSigDB data base.  
684 Molecular signatures are represented as bar-plots (FDR<0,25). (D) dHepaRG cells were  
685 infected with HDV with 50 vge/cell and treated 3 days with BS1 (0.1 µg/mL) or Pam3CSK4 (0.1  
686 µg/mL) in the presence or not of TPCA-1 (10 uM) for 11 days. Cells were collected and the  
687 levels of total intracellular HDV RNAs were analysed by RT-qPCR. Results are the mean +/- SD  
688 of 3 independent experiments each performed with three biological replicates and statistical  
689 analyses were performed using Mann-Whitney tests.

690

691 **Figure 3. Pam3CSK4 and BS1 reduced the infectivity of HDV particles.** dHepaRG cells were  
692 co-infected with with HBV (500 vge per cell) and with HDV (50 vge per cell). Cells were treated  
693 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  
694 (C) Lamivudine (3TC; 10 µM) for 10 days. Supernatants were collected, concentrated by PEG  
695 precipitation and the levels of extracellular HDV RNAs (called HDV-2P for second passage)  
696 were assessed by RT-qPCR analyses before (A, B) being used for infection of naïve HuH7.5-  
697 NTCP cells with the indicated vge per cell. Six days later, (A) levels of intracellular HDV RNAs  
698 were assessed by RT-qPCR analyses or (B) cells were stained with DAPI and with anti-HDAg  
699 antibodies. Results are the mean +/- SD of three biological replicates. (C) Concentrated  
700 supernatant were submitted to iodixanol gradients overnight. Fractions (FR) were collected  
701 and the levels of HDV RNAs, HBV DNA, HBsAg, HDV proteins were analysed by RT-qPCR, qPCR,

702 ELISA and western blot. MW: molecular weight. (D) HDV RNAs were extracted from  
703 concentrated supernatants and sequences of HDV-G RNAs from HDV-2P were analysed after  
704 RT-PCR and cloning. Bars represent non-edited HDV-G and edited HDV-G at the amber  
705 termination codon (usually edited by ADAR1 to produce L-HDAg). See figure S9 for details of  
706 the sequencing.

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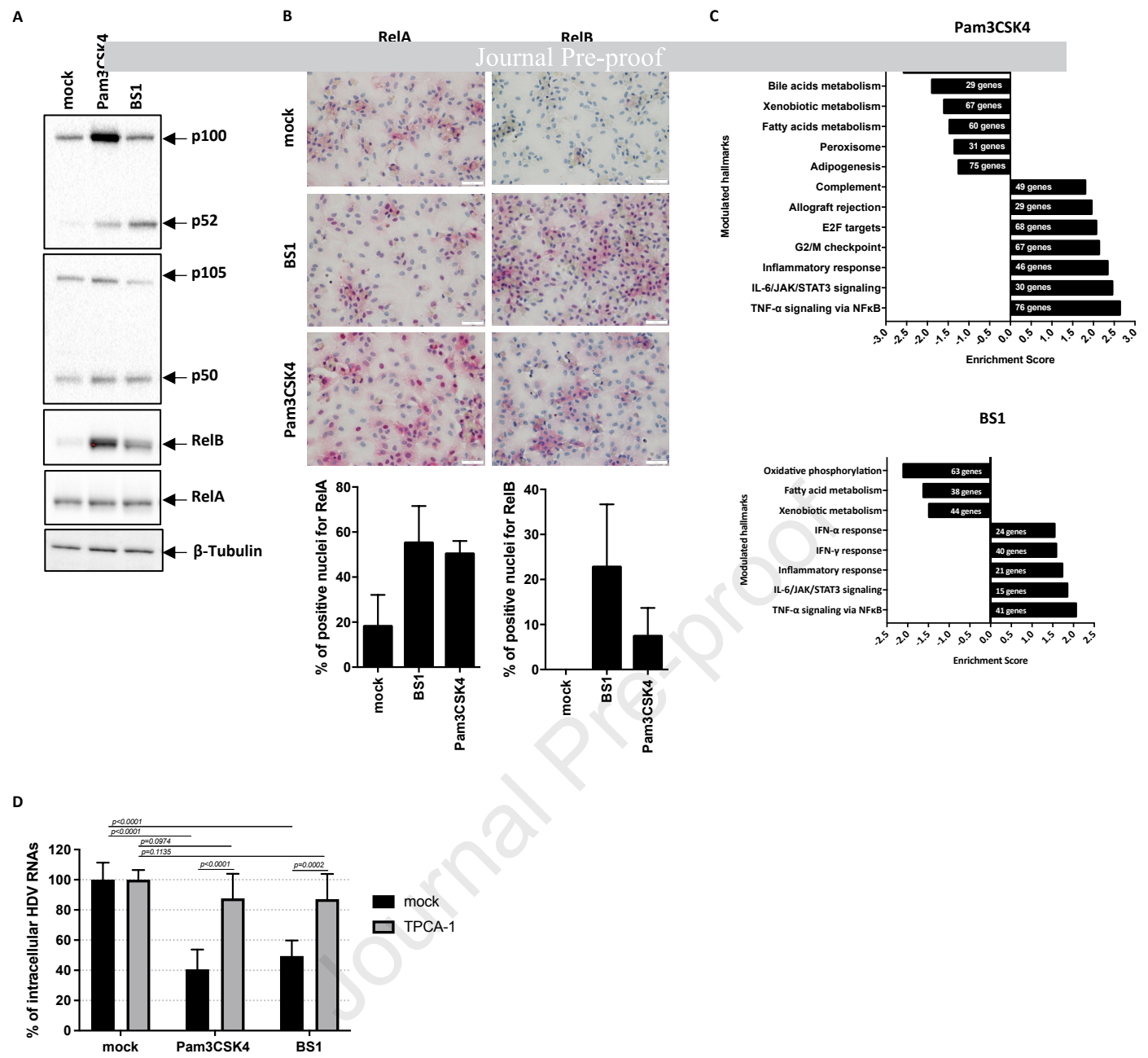


Figure 2



## 1 **Highlights**

- 2 • HDV lead to the most severe form of chronic viral hepatitis and treatment options are
- 3 limited
- 4 • We demonstrate, for the first time, that immune-modulators inducing the NF- $\kappa$ B
- 5 pathways can inhibit HDV in hepatocytes
- 6 • Pam3CSK4 (TLR1/2 agonist) and BS1 (LTBR agonist) reduced the intracellular and
- 7 extracellular HDV RNAs and proteins
- 8 • Pam3CSK4 and BS1 also strongly reduced specific infectivity of remaining HDV virion
- 9 progeny
- 10 • Pam3CSK4 and BS1 will thereby probably counter-acting viral spreading *in vivo*